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# PRINCIPLES OF MOLECULAR ONCOLOGY

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SECOND EDITION

EDITED BY

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# **Principles of Molecular Oncology**

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*Second Edition*

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## Foreword

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At the midpoint of the 20th century, our knowledge of cancer was based on epidemiology and pathology, and treatment consisted of surgery and radiation therapy. At mid-century, Medawar and colleagues initiated the understanding of transplantation immunology, Farber described the first use of an antifolic drug to treat leukemia, and Jacobson and coworkers described the irradiation-protection effect of spleen cells. These observations opened the door to the development of chemotherapy and transplantation in the treatment of cancer. Despite the rapid development of these new disciplines, progress was usually based on empiric observations and clinical trials.

The rapid advances in molecular biology at the end of the 20th century mark a new era in our knowledge of cancer. Molecular immunology, molecular genetics, molecular pharmacology, and the Human Genome Project are in the process of providing a level of understanding of cancer undreamed of in the past. Optimism is based on the firm belief that understanding at the molecular level will lead to better and earlier diagnosis, to new forms of treatment, and, most importantly, eventually to prevention of many types of cancer.

*Principles of Molecular Oncology* provides a bold new look at the evolution of our knowledge of cancer. Authors from many disciplines are bringing together the facets that provide a comprehensive view of the whole. In a field progressing as rapidly as the understanding of cancer at the molecular level, any book must be regarded as a report of work in progress. The reader will enjoy the opportunity to pause and look at the whole field as it stands today. This book will prove both informative and intellectually satisfying.

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## Foreword

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A famous London surgeon is quoted as saying that a cure for cancer would not be discovered by people in white coats working in laboratories, but rather by somebody leaning over a fence watching workmen digging a hole in the ground. Indeed, the idea that malignant disease might have a single cause was rife until quite recently. But until the era of molecular biology, and the remarkable insights into cell biology that followed, the cancer field was in the doldrums. Viruses as the cause of human cancer had come and gone, chemical carcinogens and exposure to ionizing radiation seemed to be unlikely causes of the bulk of human cancers, and it was not at all clear where to turn in cancer research. However, in the 1960s, two fields of investigation started to yield results that at least held some promise. Epidemiological studies showed quite unequivocally that there is a relationship between the development of certain cancers and cigaret smoking. And at least some forms of leukemia appeared to be associated with specific chromosomal changes. However, until the advent of recombinant DNA technology, there was no indication as to how these observations might be connected or about the cellular mechanisms of malignant transformation.

When historians of science look back on the close of the 20th century and try to evaluate the fruits of the application of molecular and cell biology to the study of human disease, it is likely that they will pinpoint the better understanding of the biology of cancer as one of the highlights of this period. The discovery of oncogenes, together with improvements in cytogenetics, resulted in an amalgamation of these two fields of research and led to the dawning of an understanding of how cancers might result from the breakdown of normal cellular homeostatic mechanisms. Subsequently, the elucidation of the genetic control of the cell cycle, and how certain oncogenes monitor different aspects of cellular activity, allowing cells to go into cycle or directing them toward apoptosis, has started to provide some insights into the cellular mechanisms of malignant disease. Almost overnight, cancer has become less mysterious. It is clear that in many cases it results from the acquisition of mutations in one or more oncogenes that we acquire during our lifetime. Since at least some of these may result from specific chromosomal changes, or from the action of environmental carcinogens, these observations provide an elegant synthesis of several different fields of research. So although the final details of how a cell becomes cancerous still remain to be worked out, at last we have a blueprint of where to go in the future.

Although it is true to say that the clinical impact of the remarkable advances in molecular medicine of the last few years may still be some time in the future, and that their immediate benefits have been oversold to the public, there seems little doubt that

these new discoveries will play a major role in the cancer field in the future. The molecular approach is likely to provide a wide range of extremely valuable diagnostic agents for both the early recognition and assessment of the prognosis of different forms of cancer. It also seems likely that gene therapy, something that has been “just around the corner” for far too long, will find some of its early applications in cancer treatment. Thus, although molecular biology has shown us that cancer is an extremely complex disease, and that there are multiple routes to the neoplastic phenotype, there is little doubt that much of this work will find application in the clinic in the not too distant future.

All these aspects of this complex and rapidly moving field are covered in this excellent book, *Principles of Molecular Oncology*. Clinical oncologists will find a series of balanced reviews of the current state-of-the-art of the diagnosis and treatment of cancer based on molecular technology, and, since cancer touches almost every field of clinical practice, specialists in other disciplines will find a very lucid and readable account of what is happening in one of the genuine success stories of today’s molecular medicine.

Writing a foreword for a book for one of one’s former students, while a constant reminder of the closeness of personal dissolution, is still an enormous pleasure. If nothing else, it is reassuring to see that at least a few resistant human lines can survive all the potential damage of medical education and emerge relatively unscathed. I wish the editors and the excellent team of authors that they have brought together all the success with this book that it deserves. In a field that is moving so rapidly it is vital to have a bird’s eye view of the state of the art: I am sure that readers will obtain a balanced view of the potential and limitations of this exciting field.

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## Foreword

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The second edition of *Principles of Molecular Oncology* is published 200 years after the exposition of Dalton's atomic theory of matter and 50 years after Watson and Crick described the basic structure of DNA. This edition comes less than four years after the first and is a consequence of the pace of discovery in such an exciting field of research. In the first edition it was anticipated that the publication of the sequenced human genome would appear in the middle of the first decade of the 21st century. It was published in 2001 and already sequenced genomes for several viruses, bacteria, plants, and animals are available. In 1844 Darwin wrote to a friend, "at last gleams of light have come and I am almost convinced (quite contrary to the opinion I started with) that species are not (it is like confessing a murder) immutable". His *The Origin of Species* issued in 1859 provided evidence for the evolutionary theory of life and represented one of the most important discoveries in biology.

The controversy surrounding Darwin's theory resulted in the famous debate between Bishop Wilberforce and Thomas Huxley. When Wilberforce finished his long tirade against the theory, Huxley replied tersely "I have come here in the cause of science only" and went on to demolish the Bishop's argument. The two men had very different backgrounds in education. Scientific method has continued to be the cornerstone in the study of life and human disease. The discovery of the structure and chemistry of DNA and the subsequent genetic research by many scientists have led to a much better understanding of the mechanisms of human biology and evolution and of the function of genes. The last 50 years has been a golden era in this important field with enormous consequences for applied medicine. Darwin of course knew nothing of genes; the processes he described were those of trial and error taking place over a vast time scale.

Recent discoveries in human genetics have not been without controversy, but clinical research has benefited from the move away from trial and error to a more rational approach in the development of new patient management techniques for many medical conditions. The techniques involved are being applied in the study of human cancer and the molecular discoveries relating to the diagnosis, prevention, early detection, and new treatments are the subject of this book. Progress in the field of molecular oncology has been much faster than previously imagined because of the abundance of innovative technology. High throughput technology for gene sequencing and expression, including comparative genomic hybridization, proteomics, and proteoglycan research, has already allowed the study of biologic function using sequenced DNA, RNA, protein, and oligosaccharide molecules. We are already awash with data and the new subject of bioinformatics has been developed to bring some order to the problem.



Poincaré, the famous French mathematician, knew from the work of Newton that the behavior of 2 bodies acting in a gravitational field could be explained with reasonable accuracy using simple mathematics but the behavior of 3 bodies was much more difficult to describe. He spent an important part of his working life on this problem and his eventual model was inaccurate. Understanding the function of genes is the key to the rational development of new treatments, but though some cancers are the result of an altered function of a single dominant gene, many arise from a more complex interaction between genes. New mathematics is being developed to help understand the complexity of these biologic systems.

In spite of the complexity, important information has been provided using molecular techniques, allowing substantial improvement in management of patients with cancer. Improvements have included the identification of predisposition to some forms of cancer, more accurate diagnostic and prognostic information, new markers for analyzing tumor progression, a quantified assessment of minimal residual disease, and the rational development of new treatments and methods of prevention. Information on all these aspects of cancer care has been updated in this new edition. It is gratifying to see that a collaborative approach between scientists in many fields is being rewarded by so much progress in the field of human cancer care. As an undergraduate at Cambridge in the 1950s, I had the advantage of contact with Crick, Brenner, Sanger, and Perutz, each of whom provided some insight into what was to come. Although since this time progress has been logarithmic, there is a great deal that remains a challenge for future editions of this book. *Principles of Molecular Oncology* provides valuable information for the continuing education of all oncologists.

**Derek Crowther, PhD, MB BChir, FRCP, FRCR**  
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Charles Darwin "Recapitulation and Conclusion," from *The Origin of Species* (1859):  
Appleton-Century-Crofts Inc.

John Dewey "The Influence of Darwinism on Philosophy," from *The Influence of Darwinism on Philosophy and Other Essays* (1910), reprinted from the *Popular Science Monthly* (July 1909), Henry Holt & Co.

## Preface

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Thomas Hodgkin's (1796–1866) criteria for determining a cancer's malignancy would still stand today: appearance of the tumor, tendency to spread, enlargement of neighboring lymph nodes, general symptoms of wasting. Until the late 18th century, medicine was symptom oriented. Toward the early 19th century, the French clinico-pathological school stressed symptoms of diagnostic significance and the primacy of physical signs. Louis Pasteur (1822–1895) did much to solve the problem of correlating microbes and disease, and Robert Koch formulated the now famous postulates to prove the pathogenicity of microorganisms. In spite of extremely important therapeutic advances (such as antimicrobials, endocrine agents, and drugs based on receptor–ligand interactions or inhibition of enzyme catalytic sites), our diagnostic skills today appear to be more potent than our ability to cure. X-rays, CT scans, NMR, ultrasounds, radioisotopes, PET scans, endoscopies, and other high-tech procedures have gradually increased our diagnostic abilities and have decreased our strict dependence on the skilled elucidation of clinical physical signs. After the important discoveries of molecular biology and genetics in the second half of the 20th century, molecular medicine is seen as the main promise for medical progress in the coming century, but it will probably come at the inevitable price of increasing complexity.

Leibniz (1646–1716) argued that Nature obeys a principle of “simplicity” or “least action.” This concept has been often associated with “positivism,” to the effect that one should choose the “simplest hypothesis” fitting the facts. Simplicity, however, has been criticized on the grounds that for any given problem there can still be several possible explanations of equal simplicity. In other words, simplicity is elegant, but it can also be deceiving. The history of science reveals progressively more complex, rather than simpler, laws and theories. In some of the most advanced sciences (for example, physics), the 20th century has brought us extremely complex theories, such as quantum mechanics or the general theory of relativity, fully understandable only to a few gifted minds.

Similarly, cancer is also turning out to be a more complex phenomenon than originally thought by many. This is why a realistic approach is a common denominator to all of the chapters of this book. Nevertheless, the search for esthetic formal simplicity and a general model pervades most of the text, together with a firm belief that even cancer can be understood and eventually defeated. The book is written by a combination of basic scientists and clinical researchers, and it is meant for practicing clinicians (such as medical oncologists, radiotherapists, hematologists, internists, general surgeons, urologists, gynecologists, thoracic surgeons, orthopedic surgeons), pharmacolo-

gists, and advanced medical students. The emphasis is not on biological mechanisms or pathology, but on prevention, early diagnosis, prognosis, and treatment.

The first chapter of *Principles of Molecular Oncology* presents the conceptual framework applicable to the rest of the book. Cancer is approached not from a specific disease-oriented point of view (e.g., lung cancer, breast cancer) nor from a selective therapeutic point of view (e.g., surgery, radiotherapy, chemotherapy). Instead, we have focused the problem starting from the hypothesis that cancer can be regarded as a “disease of key regulatory pathways.” Pathways involved, for example, in the homeostatic regulation of cell growth, differentiation, and death. Carcinogenesis, as it is understood today, involves several cumulative genetic changes that, in at least some instances, can lead to the acquisition of a malignant phenotype. At the same time, these successive molecular changes can provide us with “markers” of malignant or premalignant lesions at genetic or cellular levels or circulating in the extracellular fluids. Some can even be inherited, leading to a genetic predisposition to malignant disease. The picture is still incomplete, but it seems reasonable to propose that each individual cancer has its own natural history, genetic makeup, and clonal evolution. Each individual cancer, therefore, may provide a particular “matrix of targets” for therapeutic intervention, conditioned by the regulatory networks of the tissue of origin. Moreover, even transformed cells are liable to modulation by their own microenvironment and the immune system of the host, and therapies can be directed not only to the cancer cells themselves, but also to the immune system of the patient and the specific microenvironment (e.g., to delay or prevent angiogenesis, tissue invasion, and metastasis).

There are still many gaps in our knowledge, both in terms of biological mechanisms and new effector molecules. These “blank spaces” in the matrix will eventually be filled by rapidly accumulating knowledge, just as new atoms gradually filled the chemical periodic table at the turn of the century. It seems likely that most of these key regulatory cascades will converge into a limited number of key regulatory events: the coordinated expression or suppression of a battery of genes, the initiation of normal DNA replication at multiple different sites in the genome, the culmination of the developmental history, and cell fate, of any given clone.

The future of molecular oncology is exciting. It will have profound implications in the prevention, early detection, and treatment of cancer. It might also help us exchange some of our unhealthy life habits for healthier ones, estimate individual vulnerability to environmental carcinogens, or allow the development of effective anticarcinogenic diets. Cancers do not happen overnight, and the often protracted lag periods of cancer growth should allow opportunities for chemoprevention or new methods of screening and early detection. Nuclear magnetic devices of the future might help to detect “in vivo” areas of genomic instability or chromosomal “disorder” by focusing on abnormal DNA patterns. The ultimate outcomes of basic research (and early clinical research) are seldom identifiable while the research is in progress. Better coordination of all research efforts by university, government agencies, pharmaceutical corporations, and international scientific societies will lead to success. Medicine is evolving at a more rapid pace than ever before, with the increasing specialization and integration of parts, learning through the association of ideas and the natural equilibration of interests.

*Principles of Molecular Oncology*, and the preceding *Principles of Molecular Medicine* by Larry Jameson et al. (Humana Press, 1998) are good examples of what has already been achieved.

I am indebted to the generous contribution of all authors, from both sides of the Atlantic; and to the constant support of the other editors, and MaryAnn Foote, in particular.

Since different perspectives allow readers to arrive at their own conclusions and serve to stimulate scientific thought, we have not removed areas of controversy or overlap among chapters. We hope that this book proves useful and we invite your comments. We have tried to acquire the necessary permissions and authorizations before publication, and great care has been taken in the preparation of the chapters. Nevertheless, errors cannot always be avoided. The editors and publishers, therefore, cannot accept responsibility for any errors or omissions that have inadvertently occurred. The views and opinions expressed in the book are those of the participating individuals and do not necessarily reflect the views of the editors, the publisher, or any other manufacturer of pharmaceutical products named herein. The package insert should be consulted before administration of any pharmaceutical product.

***Miguel H. Bronchud, MD, PhD***

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# I

## MOLECULAR MARKERS

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# 1

## Selecting the Right Targets for Cancer Therapy

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**Miguel H. Bronchud**

### Introduction

For some time, many oncologists in large cancer centers have believed that better combinations of drugs would be found for treating cancer and cancer mortality would decrease, but they would not need to understand much about the mechanisms underlying the origin and spread of the disease. This rather empirical and optimistic approach led to a number of successful, albeit toxic, treatments for some uncommon cancers, such as tumors in children, several types of leukemias and lymphomas, and germ cell cancers in young men.

As stated in the first edition of this book, most clinicians believe that it will be through science and hard work, rather than magic or mere luck, that cancer will be defeated. To understand the prospects of cancer research, practicing clinicians and the public need some idea of the present state of knowledge on the subject. The problem is that in the past 2.5 decades an explosion of information, rather than knowledge, has occurred concerning the molecular aspects of cancer: >300 genes and their respective protein products have been described as directly or indirectly linked to cancer. The forest may be missed because of the trees. Cancer clinicians (including medical oncologists, radiotherapists, hematologists, general surgeons, gynecologists, and urologists) find it increasingly difficult to stay abreast of knowledge in the molecular aspects of these complex diseases. Some believe that relevant information eventually will pass from the molecular pathology laboratory to the cancer clinical units with the help of clever computer programmers. Before clinicians can decide on the curability or incurability of any given cancer and on which sequence and combination of drugs to use, they will need to consult a computer programmer and the molecular pathology laboratory.

An important objective of this second edition of *Principles of Molecular Oncology* was not only to discuss and to update the > 300 molecular markers of malignant disease (the trees), but also to continue to explain their potential clinical roles in the diagnosis, prognosis, and treatment of cancer (the forest). Several chapters are new and reflect the rapid advances in topics that only 3 yr ago were still in early infancy, including microarrays and other genomic and proteomic technologies or the use of biomarkers as surrogate end points in clinical trials or chemoprevention strategies. Other chapters

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discussing various types of tumor markers, regulatory pathways crucial for the control of cellular proliferation, differentiation and apoptosis, maintenance of genomic integrity, or new anticancer agents and promising approaches for drug development have required substantial updating.

In this chapter, I try to show the forest, rather than the individual trees. In light of present knowledge, it remains difficult to bridge the gulfs that open on closer examination, which cannot be spanned by the most audacious hypothesis. The evolution of most human cancers can be viewed as the operation of Darwinian selection processes among competing populations of dividing cells and the sequential accumulation of relevant genetic and epigenetic events. This dynamic and realistic view of cancer provides a useful background for the relevance of the numerous molecular changes that can lead to phenotypic malignant transformation and stresses the importance of early detection, the potential for chemopreventive strategies, and the eventual use of microarray technologies to detect ongoing carcinogenesis *in vivo*, and, ultimately, to lead to a new and more accurate molecular classification of cancers.

Tumor markers include genetic markers in both hereditary and nonhereditary tumors, cellular and tissue markers, and circulating cancer markers. Some of these markers are used routinely in clinical practice; several circulating cancer markers are useful for the diagnosis, prognosis, and follow-up of some types of cancer. Others are being investigated as a source of important prognostic information or even as predictors of response to chemotherapy or radiotherapy and still others are being explored in the context of genetic counseling to screen for hereditary cancer predisposition.

Regulatory pathways involved in the complex regulation of cell growth, differentiation, senescence, and cell death are being understood. The classic metabolic regulatory pathways have been known for many years. Pathways such as the citric acid cycle, the central role of adenosine triphosphate (ATP) in the energy-transfer cycles, or the intriguing hypothesis postulated to explain the mechanism of oxidative and photosynthetic phosphorylation have been in biochemistry textbooks for decades. Although the structure of DNA had been known for decades, some scientists remained pessimistic about therapeutic progress in oncology. It was difficult to visualize the discovery of new revolutionary cancer treatments, and it did not enter into the speculative, day-to-day conversation of cancer research.

This book shows that things have changed. The knowledge that has accumulated on the fine regulatory mechanisms that are deranged in cancer cells is vast and undoubtedly promises new therapeutic insights. The introduction into routine clinical use of selective tyrosine kinase inhibitors for chronic myeloid leukemia (CML) and specific monoclonal antibodies (MAbs) for breast cancer or some lymphomas are good examples. In contrast to the situation 20 yr ago, not only do we know many molecular targets for which to design new drugs for the chemoprevention or treatment of cancer, but we also have an apparent excess of targets for our current resources of drug development worldwide. The Human Genome Project completed its first basic human genome map and is likely to provide further insights and more potential targets. It is estimated that the human genome contains some 35,000 genes, fewer than originally thought. Some of these genes are well characterized and their functions in various pathways are well known. The function of most human genes remains speculative. The rate-limiting step in progress against cancer is the amount of resources we can spend, and the optimization and coordination of this huge research process, rather than a shortage or lack of therapeutic targets.

Selecting the right targets for cancer therapy can make a big difference. If we are clever or lucky enough to have correctly guessed the correct targets for the primary human cancers, and if large multinational pharmaceutical companies agree to focus their efforts and resources on these targets, then revolutionary new cancer treatments might become available for clinical testing within 5–10 yr. If we are unlucky or wrong or if not enough importance is given to this war against cancer, then it might take another 20 or 30 yr for new treatments to become available.

Four premises of cancer and cancer research are as follows:

1. Cancer can be prevented.
2. Cancer can be diagnosed; the earlier the diagnosis, the greater the chance of curative treatment.
3. Cancer often can be cured, but the impact on mortality of present therapies is limited.
4. Cancer cannot always be cured and it seems reasonable to predict that even in the year 2040, many cancers will be incurable at the time of clinical presentation.

## The Evolution of a Cancer

All cells in an organism are derived from a single cell, the fertilized egg. At an early stage in development, the embryo consists of three types of cells: ectoderm, mesoderm, and endoderm. Mesoderm develops further and part of it becomes mesenchyme. Even a relatively simple tissue such as the liver may contain many cell types. Modern gene expression–profiling methods (DNA microarrays) are good molecular witnesses of such diversity and complexity of tissues. We may never know what cancer is until the complexity of embryonic development and the precise mechanisms that regulate gene expression and DNA replication are understood, for cancer is, put in simple terms, the result of what happens when these mechanisms go progressively wrong in the adult. Cancers in the embryo are extremely rare, if they exist at all, although some neoplasms of children may well originate *in utero*.

## Relatively Old Views on Carcinogenesis

For some time, the concept of lineage has been central to cancer. According to this concept, cancer starts as a local disease in a given cell clone that, for reasons that remained unclear until some 20 yr ago, could multiply faster than normal and displace its neighbors. Described in these terms, the evolution of a cancer can be viewed as the result of Darwinian selection among competing populations of dividing cells. The tissues of the body normally preserve their initial fine mosaicism even in old age, indicating that under normal circumstances, harmony exists in tissues with equilibrium between cell death and cell proliferation, balanced regulatory exchanges between stroma and epithelial cells, and little or no competition between adjacent cells. According to this widely accepted view, one of the earliest steps in the sequence leading to cancer is the emergence of families that are able to displace their neighbors (1–3). No doubt this ability is partly the result of some intrinsic change in the cells (mutations were suspected long before oncogenes and tumor suppressor genes were discovered) that enable them to compete for territory, but the whole process can be accelerated by anything that causes cell death, creating an opportunity for competition. As a result, the normal equilibrium is lost and a tumor gradually develops.

These intrinsic changes in the cells can induce cells to multiply without the usual restraints; that is, they divide more frequently or are subject to less cell loss. The cells, however, keep within their normal territory and do not invade the surrounding tissues,

thereby forming benign tumors. Alternatively, if intrinsic changes damage the normal regulatory networks that maintain territoriality and the normal cells acquire the ability to spread to alien sites, locally or distantly, then the tumors are called cancers.

Monoclonality is considered one hallmark of tumors, but clonality is not unequivocally associated with malignancy. Clonal markers are useful for the diagnosis or follow-up of disease progression for both solid and hematologic tumors. Modern methods of clonality determination include X-chromosome inactivation (in females), immunoglobulin and T-cell receptor gene rearrangement analysis, and specific chromosomal translocations or deletions. Benign conditions (e.g., benign monoclonal gammopathy) and some premalignant conditions (e.g., lymphomatoid granulomatosis, lymphomatoid papulosis) may show monoclonal rearrangement without necessarily developing malignancy after prolonged follow-up (4,5).

Regarding the nature of these intrinsic changes that lead to cancer, several decades ago it was debated if their nature was genetic (i.e., secondary to mutations or chromosomal alterations) or epigenetic (i.e., owing to abnormalities in gene expression without an underlying genetic lesion). Although most known human carcinogens were proven to be mutagens, it was postulated that some cancers for which no cause had been discovered could be partly due to agents that were not mutagens, but acted instead by provoking cell division and errors in gene expression.

### ***More Modern Views on Carcinogenesis***

Most of the evidence is clearly in favor of a genetic cause for cancer. The incidence of cancer increases sharply with age and various models have been proposed to account for this increase (6,7). These models share the view that a cancer cell results from a series of steps that have occurred at some time in life. It has been postulated that each cell has several genes that independently restrain it from forming a family of cells, and that a cancer arises when a cell is created in which each of these genes has been inactivated by a separate, independent mutation. Logically, the probability of any particular cell having a mutation in a particular gene increases with age.

In 1958, Armitage and Doll (8) calculated that for some of the common solid tumors, the logarithm of cancer incidence should be linearly related to the logarithm of age. If their calculations are correct, then we could deduce from the slope of the death rate from cancer of the large intestine in relation to age plotted logarithmically that about six mutations are needed to produce a cancer of the large intestine. This estimate is extraordinary if one thinks that more or less the same conclusion was reached, based on molecular genetic knowledge, by Fearon and Vogelstein (9) in their classic work on colorectal tumorigenesis. The multistep mutations theory of cancer was also supported by other lines of epidemiologic evidence (10,11) and by the initiation and promotion models of carcinogenesis. A characteristic feature of most forms of carcinogenesis is the long period that elapses between initial application of the carcinogen and the time the first cancers appear. It is necessary to apply coal tar repeatedly to the skin of a mouse for several months before any tumors are detectable. Similarly, most common human cancers can take 3–30 yr or more to develop.

Experimental models of chemical carcinogenesis helped identify at least two classes of carcinogenic compounds: the initiators and the promoters. When mice are fed a small amount of the carcinogen dimethylbenzanthracene (DMBA), widespread irreversible alterations (presumably mutations) are produced. Subsequent irritation of the skin by painting it twice a week with croton oil (the promoter) eventually causes tumors. These

tumors appear even if croton oil is not started until 16 wk after the DMBA feeding, but no tumors arise if either DMBA or croton oil is given alone or if the order of the treatments is reversed. In several aspects, estrogens (for breast cancer) and testosterone (for prostate cancer) have been regarded as potential tumor promoters.

Other insights into the genetic nature of tumorigenesis came from studies on viral carcinogenesis (12–14) and from seminal observations in the uncommon retinal cancers in children (15).

The discovery of tumor oncogenes and tumor suppressor genes almost 20 yr ago led to the study of the molecular epidemiology of cancer (16–20). It soon became apparent that usually more than one somatic mutational event was needed for malignant transformation, with the possible exception of hereditary retinoblastomas, described by the two-hit model proposed by Knudson (15). It was also discovered that certain carcinogens are linked to selective mutational events. Molecular linkage between exposure to carcinogens and cancer types has been described for p53 mutational spectra of hepatocellular carcinoma, skin cancers, and lung cancer (19). Fearon and Vogelstein (9) proposed a molecular model for colorectal carcinogenesis based on the sequential accumulation of genetic events in key regulatory genes along the sequence from adenoma to carcinoma. Kinzler and Vogelstein (21) proposed the concept of two carcinogenic genetic events: those involving gatekeeper or caretaker genes characterized by their control of net cellular proliferation or maintenance of genomic integrity, respectively. Examples of gatekeeper genes include *APC* and  $\beta$ -*catenin* in colon epithelium, *Rb* in retinal epithelial cells, *NF1* in Schwann cells, and *VHL* in kidney cells. It is proposed that an alteration in *APC* leads to a rearrangement of the cellular proliferation pathway important for maintaining a constant cell population in colonic cells. Identification of other gatekeeper genes is expected, and some may be crucial to morphogenetic events of specific tissues.

Unlike gatekeeper genes, caretaker genes generally maintain genomic stability and are not involved directly in the initiation of the neoplastic process, but their mutations enhance the probability of mutations in other genes, including those in the gatekeeper class. Because multiple mutations are found in cancer cells, Loeb (22,23) suggested the existence of a mutator phenotype as an important step in tumor development. Candidate mutator genes are involved in multiple cellular functions needed for maintaining genetic stability, such as DNA repair, DNA replication, chromosomal segregation, cell-cycle control, and apoptosis.

Some individuals may be predisposed to cancer because of inherited mutations of some key genes that may confer a familial predisposition to cancer. This theory has attracted considerable attention recently, particularly in relationship to breast cancer and colon cancer susceptibility genes (24,25).

The genetic alterations in oncogenes generally lead to an increased function of the protein, whereas, in general, tumor suppressor genes are inactivated during carcinogenesis with apparent loss of function of the protein. The mechanisms of activation or inactivation are multiple, and the precise consequences on gain or loss of function are incompletely understood. *K-ras* and *H-ras* are examples of oncogenes preferentially altered by point mutation (codons 12, 13, and 61), generating a protein with constant glutamyl transpeptidase activity. *c-myc* can be activated by chromosomal translocation (in some leukemias) or by gene amplification (in some solid tumors). The *p53* and *Rb* tumor suppressor genes are often knocked out by point mutation in one allele and by deletion (loss of heterozygosity [LOH]) at the other. Other genes, such as *p16*, have high rates of homozygous deletions or promoter hypermethylation.



Some genetic defects are fairly characteristic for a given tissue type: most colorectal cancers have *APC* or  $\beta$ -*catenin* mutations. The same players are frequently involved in different tumors. Each human cancer can be regarded as a different molecular entity, with a different matrix of molecular targets, that evolves with time, even as a result of systemic or local therapies (26).

### ***Cancers are Monoclonal but the Carcinogenesis Process is Probably Polyclonal***

Progress in the physical mapping of the human genome points toward a postgenome era. Automated or semiautomated devices are capable of reading thousands of genes. Genomics and proteomics are here (27), but their routine use in the clinic requires proof of efficacy and judicious use. Apart from technical problems inherent to these techniques, some general obstacles to this promise are the following:

- Lack of genetic markers for all tumors.
- Lack of knowledge on the functional implications of most genetic defects; the precise maps of these regulatory pathways are still under investigation and may be tissue specific.
- Widespread location and heterogeneity of many of the gene mutations, particularly in large genes.
- Lack of knowledge of the prevalence of genetic defects in an apparently healthy population, i.e., people with no detectable cancer or malignant lesions.
- Lack of knowledge of the significance of each of these genetic lesions individually or in different combinations.

Cancer is a state, but carcinogenesis is a process. Key to the multistep genetic nature of cancer is that carcinogenesis is progressive. In most epithelial tissues, progression means the sequential accumulation of somatic mutations. In some cases of familial predisposition to cancer, mutations may be inherited. Gradually, a given target tissue experiences a transition from normal histology to proliferative and/or dysplastic changes, to so-called intraepithelial neoplasia (IEN), which can be early or severe, to superficial cancers (*in situ*); and, finally, to invasive disease. In some instances, the process may be aggressive and relatively rapid (in the presence of a DNA repair-deficient genotype or an aggressive human papilloma virus) but, in general, these changes occur over time. In the breast, e.g., it is estimated that progression from atypical hyperplasia through ductal carcinoma *in situ* (DCIS) to adenocarcinoma may require 30 yr (28,29). Similar observations have been made in other tissues, such as lung, head and neck, prostate, bladder, and colorectal (30–34).

It would be a mistake to believe that all common epithelial human cancers follow clear-cut histologic sequential patterns from adenoma to carcinoma. Only a few of the most common types of breast cancers (infiltrating ductal carcinomas [IDCs]) arise from DCIS. The molecular changes leading to IDC, which accounts for almost 70% of all breast cancers, can happen before the histologic features associated with DCIS become evident. DCIS is characterized by a proliferation of malignant epithelial cells confined to the mammary ducts without light microscopic evidence of invasion through the basement membrane into the surrounding stroma; however, IDC, by definition, show signs of invasion of stromal tissue, often with vascular and/or lymphatic vessel involvement. Conventional histologic and radiologic techniques (i.e., bilateral mammograms) are not enough to detect ongoing carcinogenic risk in many women at risk.

Patients with a head-and-neck squamous cell carcinoma (HNSCC) often develop multiple (pre)malignant lesions, ranging from leukoplakia to other cancers, which led

Slaughter et al. (35) to postulate the concept of field cancerization. The incidence rate of second primary tumors after a first diagnosis of HNSCC is 10–35%, depending on both the location of the first primary tumor and age. The carcinogens associated with HNSCC (alcohol and tobacco smoking) are thought to induce mucosal changes in the entire upper aerodigestive tract, causing multiple genetic abnormalities in the whole tissue region. A similar argument applies to other tobacco-related cancers, such as transitional cell carcinomas of the urogenital tract or bronchogenic carcinomas (36,37). An alternative theory for these observations is that any transforming event is rare and that multiple lesions arise due to the widespread migration of transformed cells through the whole upper aerodigestive tract (38,39). Most field changes appear to be induced by smoking, supporting the theory of carcinogen-induced field cancerization rather than field cancerization due to migrated transformed cells (40).

Other possible causes of field carcinogenic events can involve hormonal factors (changes in the ovaries, breasts, or prostate), inflammation and hyperemia (increased proliferative and angiogenic activity in chronic cystitis, gastritis, esophagitis, or colitis); chronic viral infections (hepatitis B virus [HBV] for hepatocarcinomas, Epstein-Barr virus [EBV] for nasopharyngeal carcinomas or some lymphomas), aberrant methylation linked to aging, free radical-induced DNA damage (for cancers of the gastrointestinal [GI] tract); skin exposed to ultraviolet (UV) irradiation (actinic keratosis and squamous cell carcinomas); ionizing radiation-induced damage; or aberrant morphogenetic pathways. It is possible that different carcinogenic pathways operate in different tissue fields belonging to the same organ. Adenocarcinomas of the right side of the colon are often associated with different clinical and molecular characteristics than adenocarcinomas of the colorectal region.

Even in breast cancer, the reported incidence of multicentric or multifocal lesions in areas away from the primary tumor in mastectomy specimens ranges from 9 to 75%, depending on the definition of multicentricity, the extent of tissue sampling, and different histologic techniques of examination (41–43). Multifocality or multicentricity of breast cancers may be more common than currently acknowledged.

In this context, the old field cancerization theory by Slaughter et al. (35) and the more recent multistep carcinogenesis model by Fearon and Vogelstein (9) can join in a single model: sequential field cancerization (44).

If seven sequential carcinogenic genetic hits in a single cellular clone are required for a malignant tumor to develop, it is mathematically more likely to occur in a tissue with a high background of genetic alterations in neighboring cellular clones than in a tissue with a low background of such alterations or with no detectable carcinogenic mutations (Figs. 1 and 2). The probability of a single clone accumulating seven independent but sequential genetic alterations leading to a malignant phenotype without any similar events occurring in neighboring cells is low. This simple conclusion, and the ability to measure background carcinogenesis in different parts of the body, might lead to several unexpected implications. Technology is becoming sufficiently sensitive to test the hypothesis. One potential technical problem is that in premalignant tissue, the signal (relevant oncogenetic lesions) might be diluted by noise (normal genome of most of the cells in the tissue), until the premalignant clones have expanded enough to become more numerous locally than normal cells. It is only a matter of time before this goal will be technically achievable.

A possible future objective is the development of a combined histologic and molecular staging system (Fig. 2). After a follow-up of 5–10 yr, one would expect more

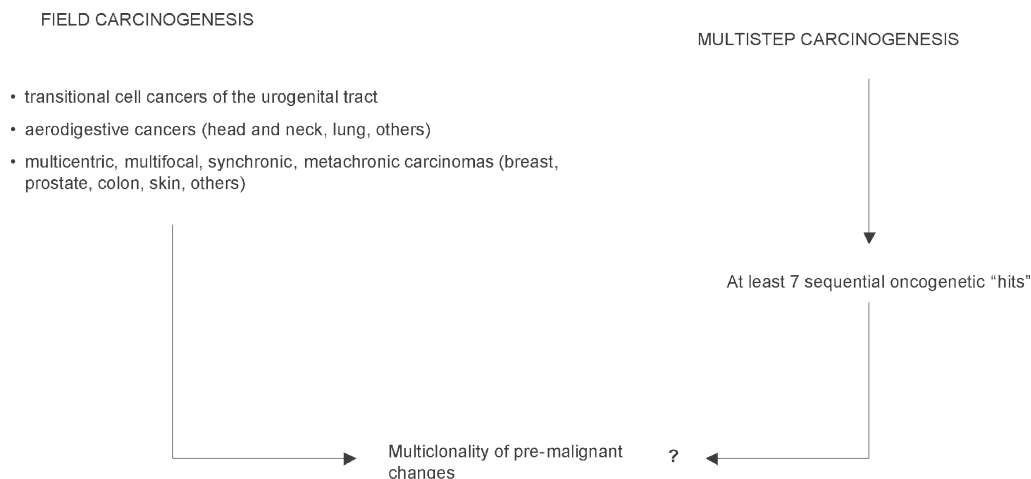


Fig. 1. Multiclonality of premalignant changes.

new cancers to develop in group IIIc of Fig. 2 (dysplastic changes and three or more significant mutations identified), than in group Ia (normal histology and no mutations identified). The clinical application of this concept and technology could classify patients into relative risk groups early in the development of a malignant disease, allowing a tailored program for follow-up and screening, and more appropriate therapeutic and chemo-preventive interventions (45–47). A suitable combination of relevant biomarkers might help clinicians identify smokers at high risk of developing lung cancers (10–15% of frequent smokers) (48,49). Confrontation with personal cancer risk rather than general statistical risk is a potent motivation to quit smoking and to undergo more frequent health checks (such as high-resolution computed tomography [CT] scans to detect isolated pulmonary nodules). Some smokers might be protected because of genetic polymorphisms of enzymes involved in the molecular activation of precarcinogens present in tobacco, whereas others might be more vulnerable to the carcinogenic effects because of genetic defects in DNA repair enzymes. Some molecular changes associated with aging and carcinogenesis might be epigenetic rather than genetic (50), and some pediatric malignancies might be secondary to abnormal morphogenetic events *in utero* (51).

The following estimates have been made for the United States:

- Thirty percent of people age 60 yr or older have adenomas of the colon by colonoscopy.
- Seventy percent of men age 80 yr or older have IEN of the prostate.
- Thirty percent of people age 60 yr or older have dermal actinic keratosis.
- Twenty percent of sexually active women age 40 yr or older have some degree of cervical IEN.
- Forty percent of heavy smokers have metaplastic or dysplastic changes in their bronchial mucosa.
- Twenty percent of women more than 50 yr of age with dense mammograms have atypical cells on ductal washings from the nipple or ultrasound-guided fine-needle aspirates.

The use of genetic or protein biomarkers relevant to each of the main cancer types may soon help better define individual cancer risks and measure background carcinogenesis in individual tissue samples. Oncology units soon may be devoted to the treatment of carcinogenesis as much as to the treatment of cancer.

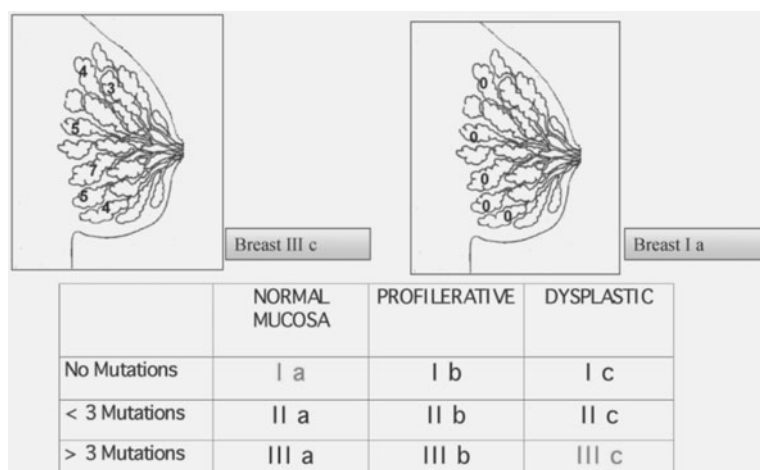


Fig. 2. Combined histologic and molecular staging system of premalignant changes. Numbers inside the drawings refer to numbers of relevant gene mutations, deletions, and amplifications identified in various regions.

DNA microarray-based sequence analysis uses comparative hybridization to obtain information ranging from mutational detection to polymorphism genotyping. Although further technical progress is needed to enable better detection of repeated sequences whose changes may not always be distinguishable from wild-type sequences, some initial experiments have found microarrays to be more sensitive, more accurate, and faster than classic sequencing approaches (52,53). New techniques are being developed to increase the detection of genomic imbalance, including gains or losses of nucleic acid material often associated with the carcinogenic process. Several groups have adapted array technology to comparative genomic hybridization (CGH), leading to so-called array-CGH techniques. In CGH, fluorescent signals along each chromosome are examined and analyzed to provide a cytogenetic pattern of gains and losses. In array-CGH experiments, the mapping resolution can reach the kilobase level (54,55). Although technical limitations remain a problem, new developments proceed at a reasonably fast pace (56,57).

Single nucleotide polymorphisms are the most frequent form of DNA polymorphisms in the human genome, with >1% differences among individuals, allowing the detection of specific genetic fingerprints, and have been used to detect LOH in several human tumor samples (58–60).

Microarray-based gene expression profiling comparisons produce a panel of upregulated or downregulated genes that can reveal candidate molecular markers for the disease in question, classify tumors into novel tumor types not previously known by conventional histologic techniques, and predict clinical outcome and response to therapies (53). Upregulation or downregulation of a gene can have genetic and epigenetic consequences, such as the gain or loss of expression of other genes or alterations in the function of several gene products, which in turn can relate to individual properties of cancer cells.

Gene expression profiling allows the identification of specific molecular fingerprints for any given cancer (61); the identification of organ-related expression of peculiar classes of genes (62); and cluster analysis of gene expression profiles for cancers such

**Table 1**  
**Matrix of Targets**

	RP1	RP2	RP3	RP4	RP5	RP6	RP7	RP8	RP9
A	N	N	LF	N	N	LF	N	LF	N
B	N	N	N	LF	N	N	LF	N	N
C	N	N	N	N	N	N	N	N	N
D	LF	N	N	LF	N	N	GF	LF	N
E	GF	N	N	N	N	LF	N	LF	N
F	GF	LF	N	N	N	N	N	N	N

The precise aberrations of regulatory pathways involved in the control of growth, differentiation, cell death, developmental history, and invasive properties can provide a matrix of targets for any given cancer. This matrix represents a molecular fingerprint of any individual cancer at a given time point, and can be used to select appropriate drugs and therapeutic strategies.

RP, regulatory pathway; N, normal gene; LF, loss of function; GF, gain of function; A–F, regulatory elements in any given pathway; RP1–RP3, growth-factor-dependent pathways operating in that specific tissue; RP4, hormone-dependent pathway; RP5, invasion and metastasis pathway; RP6, DNA repair pathway; RP7, cell-cycle regulatory pathway; RP8, apoptosis pathway; RP9, angiogenesis switch pathway.

as breast (63–65), ovarian (66), lung (67), soft-tissue sarcomas (68), non-Hodgkin's lymphomas (NHLs) (69), and prostate (70), among others.

If the precise biologic behavior of a cell depends on the precise functional status of all the main cellular regulatory pathways, then it must necessarily follow that an objective of cancer research is to use all present and future molecular techniques to define a useful picture of all regulatory pathways in any given tumor at any given time. This overall picture should provide the necessary information to determine prognosis and suitable treatment targets, as well as facilitate both target selection and *in vivo* target validation of new anticancer drugs (Table 1). The emerging discipline of proteomics may be key to the definition of this matrix of targets. Kodadek (71) has described two practical applications of proteome arrays: protein function arrays and protein-detecting arrays. The protein-detecting array consists of an arrayed set of protein ligands used to profile gene expression and draw proteosignatures of the cellular state. In protein-function arrays, a large amount of protein is spotted on a solid support and tested to characterize either a biochemical activity or a molecular interaction (protein binding, DNA binding).

Further advances are expected to come from tissue microarrays. Gene microarray analysis generally relies on the availability of fresh frozen tumor samples. These specimens are often collected prospectively and rarely have significant follow-up data associated with them. By contrast, tissue microarrays, developed by Kononen et al. (72) allow the combination of several hundred tumor specimens in a single paraffin block. Tumors can be obtained and analyzed by immunohistochemistry (IHC) for specific gene products. While they do not produce large amounts of data sets like mRNA expression profiling arrays, they generate many IHC data that require sophisticated biomathematical approaches for their interpretation.

Several laboratories are pioneering proteomic and tissue-array techniques (72–79). A new development is the combination of proteomics with conventional magnetic resonance imaging (MRI). Glioblastoma multiforme has a distinct appearance on MRI. By using MRI-guided proteomics, Hobbs et al. (80) were able to show spatial differences in protein expression patterns correlating with MRI contrast differences.

In summary, while we should not forget that relatively simple clinical and biochemical variables still remain the best prognostic indicators for most tumors (for example,

age and sex of the patient, performance status, TNM stage, circulating marker levels), or that even the psychosocial circumstances of the patient or the choice of surgeon can be relevant prognostic factors (81), it is obvious that the introduction of these new molecular techniques will revolutionize clinical oncology. It seems evident that it is too early to say which of these techniques, or which combinations of techniques, will be most appropriate to allow for the identification of carcinogenic status of any given tissue or for therapeutic target selection and validation and clinical follow-up of cancer therapies. Progress has been faster than anticipated by many, however.

## **Cancer Can Be Prevented**

### ***Trends in Mortality Due to Cancer***

About 200 distinct varieties of cancer are recognized, but some are very rare, and most cancer mortality is caused by a few cancers. In the Western world, cancer is the second largest cause of mortality after cardiovascular disease. Infectious disease, which once was the primary cause of death, now accounts for about 1–2% of all deaths. In the United States, age-adjusted mortality due to cancer in 1994 was 6% higher than in 1970. After decades of steady increases, the age-adjusted mortality due to all cancers plateaued and then decreased by about 1% from 1991 to 1994.

Changes in mortality due to cancer primarily reflect changing incidence or early detection rather than more effective therapies. The small decline in mortality due to cancer in the United States was greatest among African-American men and persons age 55 yr or older (82). These trends reflect a combination of changes in death rates from specific types of cancer, with important declines due to reduced cigaret smoking or improved screening methods and a mixture of increases and decreases in the incidence of types of cancer not directly linked to tobacco use. The use of mortality as the chief measure of progress against cancer, rather than incidence or survival, stresses the outcome that is most reliably reported and is of greatest concern to the public. Adjustment for age removes the effects of changes in the age distribution of the population, and with it the effect of changing mortality from causes other than cancer.

Trends in incidence, although important, are not quite so reliable because of the variability in the precision of diagnostic information, trends in screening and early detection, and criteria for reporting cancer. The development and commercial promotion of the prostate-specific antigen (PSA) test probably had a role in the doubling of the reported incidence of cancer of the prostate between 1974 and 1990.

In the past two or three decades, significant improvements have been made in the treatment of children and young adults with cancer, in the management of Hodgkin's disease (HD), and in the palliation of symptoms of advanced cancer (82). The effect of primary prevention (an observed trend toward a reduction in smoking in the United States) and secondary prevention (the Papanicolaou smear) are more important and support the view that the dominant research strategies of the past 40 yr, particularly the emphasis on improving treatments, should be redirected toward prevention to significantly reduce age-adjusted mortality due to cancer. These conclusions were partly challenged by other investigators (83) on the grounds that during the past 25 yr, previously fatal conditions such as advanced testicular cancer (84), HD (85), and childhood leukemia (86) have become curable in >70% of cases, and up to 50% of patients with NHL can also be cured (87). Prospective randomized trials have shown important reductions in mortality for patients with breast or colorectal cancers treated with adjuvant chemotherapies (88,89). Up-to-date long-term survival curves of

patients with cancer by period analysis, opposed to the traditional cohort survival analysis, are able to detect recent improvements in survival because of a combination of earlier detection methods and better systemic therapies (90). Cancer is likely to replace heart disease, however, as the leading cause of death in the United States within the next few decades, and in some developed areas (such as Catalonia), cancer deaths are already higher than cardiovascular deaths in men aged 35–75 yr. This situation is partly due to population screening and suitable therapeutic or prophylactic intervention (both by pharmacologic means and by changing lifestyles) for cardiovascular risk factors, such as obesity, high serum cholesterol levels, diabetes, hypertension, and sedentary lifestyle.

It seems prudent to conclude that not enough money is spent on cancer research worldwide. In spite of the US \$3.7 billion through the federally sponsored National Cancer Institute programs, the amount is negligible in comparison to the reported US \$100 billion spent on the war on terrorism, particularly if one considers that more than 10,000 Americans and similar numbers of Europeans die from cancer each week (91). Of course, the legitimate defense of our freedoms and values deserves adequate investments and determination, but the fight against cancer is a daily war that is much closer to us than we think until this often deadly disease affects us, a close friend, or relative. Unfortunately, both freedom and health are key values to our human existence that are too often missed only when they are lost or threatened. In this respect, it is encouraging that President George W. Bush has promised to increase funding for cancer research by US \$629 million for the 2003 budget, reaching a total investment in cancer research of more than US \$5 billion through the National Institutes of Health (92). President Bush acknowledged that 1500 citizens die of cancer every day and that three of every four families in the United States will have at least one family member diagnosed with cancer. At the same time, a report by the consumer health organization Families USA has stated that eight of the nine top companies that market most of the common drugs used in oncology have spent more than twice as much on marketing, advertising, and administration as they did on research and development (93).

The long latency periods that commonly occur between the first exposure to the carcinogenic agent and the appearance of clinical disease should be seen as a window of opportunity for early diagnostic and therapeutic intervention and possibly cure. This latent period usually lasts 20–40 yr, although it may be as short as 1 or as long as 60. The interval is subject to random factors, partly because few cancers are induced by a single brief exposure and partly because relatively few data exist with detailed reliable information about the exact dates when exposure began and ended. After the Hiroshima and Nagasaki nuclear bombs, the peak incidence of leukemia occurred about 5 yr after exposure, but the incidence of solid tumors increased for at least 15–20 yr. The evidence that many of the common cancers are preventable can be derived by four groups of observations: differences in the incidence of a particular type of cancer between different settled communities, differences among migrants from a community and those who remained behind, variation with time within particular communities, and actual identification of a large number of specific and controllable causes (94).

### ***Diet and Cancer***

Diet is often blamed for about 30% of human cancers, and epidemiology and laboratory research are needed to understand the important causes of cancer and learn how to prevent it. With at least 10,000 trace chemicals detectable at one time or another in our bodies, the task of relating cause and effect is not easy.

For several decades, evidence suggested that many of the common cancers could be made less so by modification of diet. With few exceptions, little reliable evidence exists as to what modifications would be of major importance. Epidemiologic data suggest that people with diets rich in  $\beta$ -carotene have lower cancer rates, particularly lung cancer rates, than people with  $\beta$ -carotene-poor diets. In the  $\alpha$ -tocopherol,  $\beta$ -carotene cancer prevention trial (ATBC) and the  $\beta$ -carotene and retinol efficacy trial (CARET), both of which included smokers, more new cancers and deaths were seen in the treatment groups than in the placebo groups. Relatively recent studies in animal models (95) suggest that a diet very rich in  $\beta$ -carotene may be enough to promote squamous metaplasia in the lungs, a precancerous stage, probably because of increased expression of tumor promoters such as c-jun and c-fos, and decreased expression of tumor-suppressor retinoic acid receptor- $\beta$ . This example is a good illustration of the difficulties associated with any experimental manipulation of diet.

A number of peculiar findings in animal models, reported and magnified by the popular press, have led to exploitation in the media, including the relationship between cyclamates and bladder cancer, between coffee and pancreatic cancer, and between potato chips and cancer. A report by the Environmental Chemistry Department of Stockholm University indicated that an ordinary bag of potato chips may contain up to 500 times more acrylamide than the maximum concentration allowed by the World Health Organization (WHO) for drinking water (96).

Consumption of alcohol has been shown to potentiate the effects of tobacco smoke on cancers of the mouth, pharynx, esophagus, and larynx (97). In combination with smoking, the risks can multiply 35-fold among heavy consumers of both products, and the amount of cancer associated with alcohol could be higher than the 3% previously estimated.

The retinoids, synthetic and natural derivatives of vitamin A, are agents active in cancer therapy and prevention. Advances in the understanding of nuclear retinoid receptors or coregulators that transmit the appropriate growth and differentiation signals (98) have led to hopes that diet supplementation with the adequate types and amounts of these substances may contribute to chemoprevention of cancer (99).

A consistent finding in epidemiologic studies on cancer and diet is the protective effect of fresh fruits and vegetables. Besides carotenoids, some studies have suggested that vitamins C and E and allium vegetables may protect against stomach cancer (100), whereas intake of fiber may lower the risk of colon cancer (101).

If one looks at the independent scientific evidence, it can be reasonably concluded that residents of the Western world eat too much animal fat. The amount of fat and saturated fat consumed in foods by the average citizen of the United States or Northern European countries (Germany, Great Britain, and Holland) is dangerously high. The incidence of heart disease is increasing in these countries. Great Britain, where animal fat consumption is high, has an exceptionally high heart attack rate.

Fruits and vegetables, which contain several important antioxidants, are generally believed to reduce the risk of stomach cancer (102). A randomized trial in a high-risk area of Colombia (103) with vitamin C supplementation at relatively high dose (2000 mg/d) showed a significant regression of precancerous stomach lesions. A very large (>1 million individuals) cohort study in the United States suggested that the use of vitamin C, vitamin E, or multivitamin supplements, regardless of duration of use, however, may not substantially reduce risk of gastric cancer mortality in North American populations (104). Although these negative results do not rule out positive effects of



vitamin supplementation in geographic areas in which stomach cancer rates are very high, they constitute another example of disappointing results in cancer prevention by dietary interventions.

Cancer mortality rates and incidence show great variability across the world (105,106). One of the best examples is malignant melanomas, uncommon in sub-Saharan African populations and common among white Australians of British descent. Undoubtedly, both genetic and environmental factors play a major role. The implication of specific metabolic susceptibility genes or population-based genetic polymorphisms in cancer risk has often led to contradictory or even negative results (107). Terry et al. (108) found no significant association between breast cancer risk and the Western dietary pattern, characterized by such foods as red and processed meats, refined grains, fats, and excess sweets compared with the supposedly healthier diets rich in fruits, vegetables, fish, poultry, and whole grains.

Fraumeni (109) admitted that in view of the limitations of nutritional methods in epidemiology, further progress is very likely to depend on innovative analytical studies using biochemical assays, as well as intervention studies involving dietary supplements and modifications.

### **Viruses and Cancer**

Another example of the potential preventability of cancer comes from virus-related carcinogenesis. It is intriguing that nearly all known oncogenic retroviruses, key to the experimental identification of many oncogenes, affect species other than humans. Several clear-cut examples of human viruses as carcinogens are known.

About a century ago, cancer was more frequent in women than in men in nearly all countries owing to the great frequency of carcinoma of the cervix uteri and the rarity of smoking-related cancers in women (such as lung, bladder, or head-and-neck cancers), and it is still more common in populations in several Latin American countries where these conditions still hold. Elsewhere, because of population screening in sexually active women for cervical cancer in developed countries, with Papanicolaou cytologic smears and colposcopic and hysteroscopic techniques, cancer is now more common in men. Cervical cancer in women is still prevalent worldwide, and it is the leading cause of cancer mortality and morbidity in countries such as Mexico, Colombia, and Ecuador.

The evidence is very strong that human papilloma viruses (HPV) are etiological agents associated with most types of cervical cancer (both squamous and adenocarcinomas). Several large cohort studies have consistently shown that HPV infections precede the development of cervical cancer by 10–15 yr. Besides HPV DNA detection, additional markers of carcinogenic progression include HPV type, estimates of the viral load, persistence of viral infection as determined by repeated sampling, viral integration into the DNA of the host cell, and other environmental factors, such as smoking and other sexually acquired infections (110–112).

HPVs are small DNA viruses that usually cause dermal warts, but can also infect the genital tract. More than 30 HPVs have been identified in the female genital tract, and 4 of these (HPV-16, HPV-18, HPV-31, and HPV-45) account for 80% of cervical cancers and code for at least two oncogenes (E6 and E7) that are expressed once the viral genome integrates into the host's DNA, disrupting some of the key pathways of cell-cycle control and apoptosis. Studies suggest that the ideal viral protein for therapeutic intervention in cervical precancerous or malignant lesions is probably the E6 polypeptide because a key

element is the induction of host apoptotic pathways, thereby eliminating a primary viral infection as well as the virus-transformed cancer cell (113). E6 binds to E6AP, the prototype HECT domain protein, and forms an E3 ubiquitin-protein ligase that ubiquitinates p53 resulting in rapid p53 degradation by the 26S proteasome (114).

The use of antisense oligonucleotides against E6AP (115) or of small peptides to block the activity of E6 (116) to degrade p53 might be valid therapeutic options, provided that the p53 pathway leading to growth arrest or apoptosis is still functional or can be reactivated in infected cells. Not only does a particular molecular target matter, but the global state of regulatory pathways in any given cancer, which we have called, in the past, the matrix of targets, matters also (117) (Table 1).

The detection of HPV infection using molecular diagnostic methods such as the polymerase chain reaction (PCR) is used together with the traditional cytologic Papanicolaou smear analysis in screening programs of early detection of cervical cancers. Theoretically, HPV vaccines should be able to prevent infection and protect against the malignant transformation. No effective vaccines have been developed, partly because of the multiple serologic subtypes and partly because the main protective immunity agent active in mucosal membranes (such as the cervix uteri) is immunoglobulin A, which is only temporarily induced so that a putative HPV vaccine would need to be administered repeatedly to maintain an effective level of immunity. A live attenuated virus vaccine, however, modeled on the successful control of smallpox, yellow fever, and polioviruses, could offer several advantages: infection of the female genital tract might confer long-lasting immunity similar to that following natural infections and could prevent reinfection. In addition, live attenuated viral vaccines are good candidates for inexpensive mass immunization in underdeveloped countries (where the prevalence is highest) without the need for sterile equipment. A potential problem is that cohort human population studies suggest that infection with one type of HPV may not confer protection against other types (118,119).

The first report of a successful vaccination program for HPV type 16 has been published (120). In this double-blind study, 2392 women were assigned to either placebo or vaccine. The incidence of persistent HPV-16 infection was 3.8 per 100 woman-years at risk in the placebo group, and 0 per 100 woman-years in the vaccine group (100% efficacy).

Costs of anticancer vaccination programs are likely to increase dramatically. HBV vaccination has been called the world's second best cancer control program, after the campaign against cigaret smoking. The association between hepatocellular carcinoma and HBV in some countries such as Taiwan and Gambia is very strong. Implementation of HBV vaccination programs in neonates and children has already reduced, according to WHO figures, the occurrence of hepatocellular carcinomas in the first decade of life. In contrast to HPV, no evidence exists that the HBV genome carries a true oncogene, and the carcinogenic activity of the virus is thought to be due to indirect effects through chronic liver damage: hepatocellular injury, necrosis, inflammation, and liver degeneration.

EBV is a highly B-lymphotropic virus, targeting B-lymphocytes through a specific interaction between their major envelope glycoprotein and a complement receptor molecule. It is an herpesvirus widespread in human populations and is carried by most individuals as a asymptomatic lifelong infection. The virus, however, is associated with several malignancies, including endemic Burkitt's lymphoma; nasopharyngeal carcinomas; the midline lethal granulomas (a particularly aggressive form of NHL);

many cases of HD; and a relatively new entity, posttransplant lymphoproliferative disease (121). The expression of viral antigens in EBV-positive HD raises the possibility of developing tumor immunotherapy. Virus-related malignancies are, at least theoretically, ideal targets for immunotherapy because the viral proteins in the tumor cells provide unique targets for antibodies or cytotoxic T-lymphocytes (CTLs). Autologous EBV-specific CTL infusions have been shown to be safe and to protect bone marrow transplant (BMT) recipients from posttransplant lymphoproliferative disease (122).

An interesting, nonviral association between an infectious agent and cancer is the case of *Helicobacter pylori* infection. The eradication of *H. pylori* leads to remission induction in most patients with low-grade gastric mucosa-associated lymphoid tissue (MALT) lymphomas in limited stages, which supports the view that *H. pylori*-induced gastritis somehow leads to clonal evolution and malignant transformation of CD5 and CD10 B-lymphocytes (123). After removal of the underlying stimulus (*H. pylori*) by appropriate antibiotic therapy, however, an ongoing process of somatic hypermutation of B-cells and antigen selection can be detected, challenging the view that these lymphomas can be entirely cured by the elimination of *H. pylori* infection (124). The current hypothesis is that the greatest risk of developing gastric lymphomas as a consequence of *H. pylori* infection is seen when there is a tendency to increased inflammation and decreased capacity to cope with the ensuing oxidative stress.

### **Nicotine and Tobacco**

Smoking is so widespread that it is rarely viewed as a form of drug abuse or as an addiction, even though it fits all of the accepted criteria for drug dependence (125). The potential adverse effects of tobacco use, unlike other addictive disorders, are associated with chronic rather than experimental or occasional use. So much has been written on the links between smoking and serious health problems (chronic respiratory problems, cancer, and cardiovascular disease) that repetition would be tedious. If there is one carcinogen whose importance has been unshakably established, it is cigaret smoke. It has been estimated that if smoking were abolished, the number of people dying of lung cancer would decrease by 80%. It has been calculated that a person's life is shortened 14 min for every cigaret smoked. Despite public awareness of the serious health consequences of smoking, the worldwide incidence and prevalence of cigaret smoking is increasing. In some regions, the number of women who die of smoking-related cancer of the lung is higher than the number of women who die of breast cancer. Although considerable evidence exists that nicotine is the reinforcing constituent that gives tobacco its universal popularity, it is still uncertain why it creates so enduring a pattern of self-administration. Smokers may continue to smoke for enjoyment or social reinforcement, to alleviate anxiety, or to be fashionable: environmental stimuli and social reinforcers interact with pharmacologic factors. It is clear that the rate of relapse among compulsive smokers (who report irritability, depression, and autonomic function changes when they give up smoking) is discouragingly high, with only 10–25% not smoking 2 yr after initial cessation efforts.

Considering that tobacco smoking is the main environmental risk factor for lung cancer, but only some 10% of heavy smokers actually develop a cancer of the bronchial epithelium, genetic polymorphisms may modify the individual susceptibility to lung cancer through their roles in metabolizing tobacco procarcinogens. *N*-Acetyltransferase-2 (*NAT2*) and microsomal epoxide hydrolase (*mEH*) are polymorphic genes that metabolize different tobacco carcinogens. A well-designed study has shown that

*NAT2* rapid acetylator genotypes are protective against lung cancer in nonsmokers, but are risk factors in heavy smokers. The joint effects of *NAT2* and *mEH* polymorphisms are consistent with an independent, additive effect of these two genes, modified by smoking history (126). The linear relationship between pack-yr of smoking and the odds ratio of lung cancer risk remains valid.

A vaccine against nicotine might induce an immune response (cellular and/or humoral) with both specificity and memory. An adequate antibody response to nicotine might neutralize its pharmacologic properties and abolish the reinforcing constituent responsible, at least in part, for addiction to tobacco smoking. Alternatively, a cellular immune response might induce a local inflammatory reaction in the lips, tongue, or oropharyngeal mucosa and discourage people from smoking. Nicotinic esters can produce nonimmunologic contact urticaria (127), at least in some animal models, but the potential allergenic nature of nicotine has not been fully investigated.

The prospects for immunotherapy as a new tool for smoking cessation are increasing, following experiments in rats (128) that provide proof of principle by showing that nicotine-specific antibody can prevent the reinstatement of nicotine self-administration. A phase 1 trial of a human cocaine vaccine has been successfully completed (129), and a safe and effective human nicotine vaccine would potentially have fewer side effects and better compliance than existing smoking cessation pharmacologic treatments (130). Stereospecific nicotine receptors can be measured on brain membranes (131). Within the last 5 yr there has been, in the United States in particular, a large increase in smoking cessation research, encouraged by the release of the 1996 Clinical Practice Guidelines for Treating Tobacco Use and Dependence (132). Several efficacious treatments exist that can double or triple the likelihood of long-term cessation for adult smokers (133).

### ***Hereditary Predisposition to Cancer***

Genetic aberrations and family clustering have been described for almost every tumor type, and a comprehensive family history should be part of the assessment of all patients with cancer (134).

Breast cancer and colon cancer families represent the majority of consultations at familial cancer clinics. Mutations in *BRCA1* and *BRCA2* are associated with a strong predisposition to breast cancer in women; according to some estimates, a gene carrier in a high-risk family has a 50% chance of developing breast cancer by the age of 50, and up to an 80% chance by age 75. It is likely that these risks are to some extent population dependent and are modified by both genetic background and environmental factors. In addition to female breast cancer, mutations of *BRCA1* also confer a substantially increased risk of epithelial ovarian cancer. Mutations in *BRCA2* confer, in addition to female breast cancer, a significantly increased risk of male breast cancer. The genes are very long (*BRCA1* has 5500 bp; *BRCA2* has 11,000 bp), and mutations can occur anywhere along their length, which makes mutation analysis very difficult, technically demanding, and expensive (135). A specific region in exon 11 of the *BRCA1* has been associated with greater ovarian risk.

The situation is different in familial adenomatous polyposis (FAP), for which current laboratory testing is said to identify mutations in about 80% of individuals with the disease (136). The failure to identify a mutation in an individual with clinical FAP should not alter the diagnosis nor the recommendation for surveillance and prophylactic colectomy. The APC protein is a rather important intracellular protein associated

with catenins, which bind cell-surface cadherins and appear to regulate cellular adhesion in the crypts of the colonic mucosa where the proliferation rate is the highest.

The Lynch syndromes, or hereditary nonpolyposis colon cancer (HNPCC), account for a greater proportion of hereditary colorectal cancer families than FAP (137–139). The Amsterdam criteria for HNPCC were a bit restrictive: three individuals with colorectal cancer in a kindred, at least two in first-degree relatives and at least one diagnosed before age 50. The genes responsible are called mismatch repair (MMR) and were identified because of the observation of replication error repairs (RERs) in the tumors of mutations carriers. Several genes are responsible (*hMSH2*, *hMLH1*, *hMSH6*, *hPMS1*, *hPMS2*). They are usually large and many mutations are of unclear significance. Measuring the RER phenotype may be a reasonable way to begin in many families because it is less expensive than direct genetic testing and it is detectable in about 80% of HNPCC tumors. Unfortunately, it is not widely available and RER techniques are not standardized across diagnostic laboratories.

The Li–Fraumeni syndrome requires at least three cancers for its formal definition, but the associated p53 germline mutations are not uncommon in individuals with soft-tissue sarcomas and adrenal cortical carcinomas, regardless of family history. Nearly 50% of women with Li–Fraumeni syndrome develop breast cancer, but this disorder probably affects <1% of all patients with breast cancer.

It was thought that the same genes were involved in the genesis of both inherited and sporadic cancers, but it has been shown that sporadic or acquired breast and ovarian cancers have mutated *BRCA1* or *BRCA2* <10% of the time. Thus, lessons learned from hereditary cancer susceptibility are not necessarily relevant to sporadic cancers. It is evident that there are more, yet unknown, breast cancer susceptibility genes in breast cancer families (140).

The link between ataxia telangiectasia (AT) and breast cancer is controversial. AT is a relatively rare autosomal recessive disorder characterized by a progressive cerebellar ataxia with onset in early childhood, cutaneous telangiectasia, increased sensitivity to ionizing radiation, and susceptibility to lymphoid malignancies. A gene in chromosome 11q22–23 is mutated in patients with AT, the *ATM* gene. The protein product is probably related to signaling cell death by apoptosis in response to DNA damage. Carriers (estimated at <1 in 200 in the British population) have, according to some studies, a two- to four-fold increased risk of breast cancer (141), but other studies deny a contribution to early-onset breast cancer (142,143). *ATM* is also an important tumor suppressor in B-cell chronic lymphocytic leukemia, and there is some suggestion that it might share a common pathway with p53, by which damaged cells are prevented from dying through apoptosis (144).

The American Society of Clinical Oncology (ASCO) has endorsed genetic testing as a component of a comprehensive cancer risk assessment, but not population-based genetic screening, which is unwarranted given the limitations of currently available tests (145,146). The clinical utility of genetic testing should be determined through adequate long-term longitudinal follow-up studies (147). Predictive testing for HNPCC or breast cancer in presymptomatic individuals is not void of ethical and psychosocial problems (148–151).

### ***Implications for Screening and Chemoprevention***

Cancer chemoprevention can be defined as the treatment of carcinogenesis, its prevention, and its inhibition or reversal (152). The term *chemoprevention* is controver-

sial. *Chemo* may lead to a confusion with chemotherapy, and *prevention* may not be the best word to define the early detection of cancer biomarkers. The subject is bound to grow very rapidly, both in terms of the identification, validation, and clinical relevance of cancer biomarkers, and in terms of their impact on the quantitative estimation and prediction of individual human cancer risks.

It has been suggested that pharmacologic agents or nutritional modification might prevent the development of human cancers or at least slow progression. The concept of chemoprevention is gaining support, although significant experimental and conceptual problems are associated with it. If the study population comprises normal or nearly normal subjects (normal volunteers, smokers without obvious disease, or people with a history of one isolated GI polyp), very few side effects may be acceptable. By contrast, if the subjects are at high risk of cancer (have a history of FAP, hereditary predisposition to breast or colon cancer, or second tumors), considerable side effects may be acceptable.

Certain metabolic or detoxification phenotypes that may confer a higher cancer risk, such as aryl hydrocarbon hydroxylase (AHH) activity, debrisoquine hydroxylation and glutathione-S-transferase activity are also of scientific interest. AHH activity depends on one subfamily of cytochrome P-450 microsomal enzymes that convert polycyclic aromatic hydrocarbons into carcinogenic intermediates (153). Case-control studies have yielded contradictory results regarding the association between AHH activity in lymphocytes and lung cancer risk (154). The reasons why only a few heavy smokers develop lung cancer remain largely unknown.

$\beta$ -Carotene, the retinoids, folic acid, vitamins C and E, and tamoxifen have been reported in published phase 3 clinical chemoprevention trials. The retinoids have been studied the longest and, although their therapeutic index is somewhat narrow because of toxicities, they have been successful in oral leukoplakia (155), actinic keratosis (156), and prevention of skin cancer in xeroderma pigmentosum (157), and second primary tumors in HNSCC (158). More recent, but rapidly growing, is the role of other agents, such as cyclooxygenase-2 (COX-2) inhibitors in chemoprevention. COX-2, a key enzyme for the production of prostaglandins from arachidonic acid, is overexpressed in colon carcinogenesis.

The general press and ASCO have reported the role of tamoxifen in chemoprevention of breast cancer (159). Tamoxifen, at 20 mg/d for 5 yr, may reduce risk to women with a defined breast cancer risk of  $\geq 1.66\%$ . Risk/benefit models suggest that the greatest clinical benefit with the least side effects is derived from use of tamoxifen in younger premenopausal women who are less likely to have thromboembolic sequelae and uterine cancers. Data confirm a substantial reduction in breast cancer risk but do not yet show an overall health benefit or increased survival. Tamoxifen is not free from side effects, but health-related quality of life is not adversely affected in most cases (160). These trials are ongoing and recommendations will be updated regularly.

### **Cancer Can Be Diagnosed and the Earlier the Diagnosis, the Higher the Chances of Curative Treatment**

More than 90% of human cancers arise in epithelial cells (carcinomas) and most of them originate in our surface epithelia (skin, respiratory tract, and gut) or secondary sexual organs (prostate and breast), suggesting some kind of interaction between carcinogenic agents that cannot penetrate very far and tissues with a high rate of cellular proliferation or hormone dependence. Less than 10% of the cancers arise in the supporting tissues of the body or the circulating cells (sarcomas and leukemias). Cancer is primarily an aging-

related condition and almost 60% of all cancers occur in people aged 65 or older (>80% for prostate cancer, 74% for colon cancer, and 72% for pancreatic cancer).

Current diagnostic skills may be no better than current therapeutic skills. In particular, the advent of diagnostic techniques such as ultrasound, CT scanning, and nuclear magnetic resonance, together with cytology (fine-needle aspirates) and IHC methods, can allow the detection of small primary or secondary tumors (measuring <1 cm<sup>3</sup> in diameter) anywhere in the body, and emerging molecular and cytologic techniques allow the detection of precancerous lesions. Exciting new technologies are allowing the in vivo monitoring of metabolism of cancer cells, or drug action by MRI (161), antisense imaging (162), and positron emission tomography (PET) (163). PET has great promise in the assessment of response to treatment. The conventional structural-based imaging techniques, such as CT, endoscopy, and endoscopic ultrasonography, are generally considered inaccurate in this setting because these modalities cannot reliably differentiate treatment-induced necrosis, fibrosis, and inflammation from residual or recurrent tumors. However, PET scanning is being increasingly used for the assessment of response to induction radiochemotherapy in a variety of settings, and the degree of concordance between the response assessment by PET and histopathology is often >75% (164). It is hoped that these new techniques will eventually enable both targeted and nontargeted therapies to be efficiently evaluated in patients, without the need for invasive techniques.

Since the publication of the first edition of this book, the public's perception of the importance of mammography screening underwent a drastic change as a consequence of the publication of article extremely critical of mammographic screening (165). This article prompted review of earlier data and ideas (166–168) and led to the conclusion that participation in mammography screening resulted in at least a 50% decrease in breast cancer in Sweden (169). Randomized clinical trials often measure the effect of invitation to screening, rather than the effects of participation in screening, since the overall results are weakened by nonparticipation of invited women and mammographies in noninvited women (170,171).

As in the case of breast cancer, improvements in prostate cancer screening methods, and particularly the introduction of PSA testing and reliable prostate ultrasound methods, have led to increased detection of early lesions. As stated by Boyle (172), "it is difficult to imagine a more controversial issue in public health or oncology at present than whether testing for PSA should be widely applied as a screening test for prostate cancer." PSA screening for prostate cancer is gaining popularity around the globe. The consequent increase in localized forms of prostate cancer has reawakened a long debate about which primary local therapy is best (whether radical prostatectomy or radical radiotherapy), as well as the relevance of adjuvant or neoadjuvant endocrine therapies. The two leading hypotheses for the association of local treatment failure (i.e., persistence of local malignant disease) are as follows: that the increased risk of metastasis is due to the inherent biologic virulence of the cancer (now beginning to be assessed also by expression profiling of tumor mRNA); and the reseeding theory that simply states that failure to completely eradicate the tumor may lead to subsequent shedding of tumor cells and a late wave of metastases. Both theories are compatible (173). After definitive radiation therapy, repeat biopsy series have shown that a sizable proportion of men without clinical evidence of persistent disease have histologic evidence of residual cancer in the prostate, with positive repeat biopsy rates ranging from 14 to 91% (174). Data from a nonrandomized, single-center study (175) suggest

similar clinical outcomes from either radical prostatectomies or radical external-beam radiotherapy for localized prostate cancer. Eight-year biochemical failure rates were identical and outcome is determined mainly by pretreatment PSA levels; biopsy Gleason score; clinical T stage; and radiation dose, if applicable. If the decision to treat implies radiation rather than surgery, higher-than-conventional radiation doses given by the most modern techniques are the preferred treatment for localized prostate cancer, but adequate clinical and PSA follow-up are required (176,177).

Considering that prostate cancer is driven by androgen, the androgen receptor (AR) is an ideal target for chemoprevention of prostate cancer. The ability of the agent finasteride to prevent prostate cancer is being studied in the Proscar Study, a large phase 3 study that has enrolled >20,000 men. Final reasonably mature results of this study are expected to become available soon.

An example of another common cancer that if detected early can be easily cured is colorectal cancer. Despite advances in diagnosis and therapy, long-term survival for this disease has not improved significantly over the last few decades, and almost 50% of patients will eventually die of their disease. Fecal occult blood testing (FOBT) was proposed for screening in the 1970s when fiber-optic endoscopic techniques were in development. Despite large clinical trials, FOBT screening has shown only a modest impact on mortality from colorectal cancers, is not well accepted, and can miss >50% of cancers (178). By contrast, endoscopic techniques detect >90% of cancers and large polyps within reach of the endoscope (179). Several prospective studies suggest that colonoscopy combined with polypectomy may result in a 66–90% decrease in cancer incidence after 6–13 yr (180–182). Colonoscopy is often presented as an expensive technique, with low acceptability by the public, and not without serious risks. In expert hands, however, routine colonoscopy has an overall complication rate of only 0.3% (perforation, bleeding of removed polyp, side effects due to sedation), and if colonoscopy costs are US \$750 or less, one-time colonoscopy is more effective than any other screening program at every level of compliance (183,184).

Compliance for colonoscopy can be increased by promoting better health education and public awareness. Newer radiologic CT scan techniques have allowed the development of the virtual colonoscopy (185), which, although more likely to be acceptable to the public, cannot provide tissue sampling or allow therapeutic polypectomies.

It is becoming clear that, for unknown reasons, distinctive and highly significant differences exist in the patterns of gene expression distinguishing right side from left side colonic normal and malignant mucosa (186). The colon can be regarded, from an anatomic point of view, as a single organ, but from biologic and molecular points of view it consists of at least two tissue fields. A review of the updated American Cancer Society guidelines for the early detection of prostate, colorectal, and endometrial cancers can be found in ref. 187.

The case of early detection and screening for lung cancer, which in the United States alone leads to an overall mortality far superior to any other cancer, is more complex than those of breast, prostate, or colorectal cancers. In 1987, the Japanese government instituted an ambitious program for lung cancer screening using chest radiographs and sputum cytology in >7 million smokers and, more recently, pioneered the use of low-dose helical CT scanning (188). In a large Japanese study (189), 1611 asymptomatic people aged 40–79, 86% with smoking history, were screened by low-dose helical CT scans, chest radiographs, and sputum cytology. In 14 cases (0.87%), lung cancers were detected by the helical CT scans, with 71% being curable stage IA disease.



Aging is a normal biologic process that leads to senescence and eventually death. From the point of view of the species, after individuals have reached the peak of their sexual maturity, they can decline in their optimal physical state to allow the next generation to grow. This decline in body function (and total body water) is what we call aging and is a uniform and gradual loss of full vital fitness and faculties. For example, loss of elasticity is a general and uniform phenomenon, but most humans will have not only from this uniform type of aging, but also a patchy tissue aging that can result in disease. Field cancerization (in smokers, in the oral mucosa, the bronchial tree, and the urinary bladder) and atheroma formation (in the aorta at different levels and bifurcation in patients with hypercholesterolemia) will eventually lead to cancer and cardiovascular disease. These scattered and patchy processes do not happen overnight but can take many years (on average >10 to 20) to develop into clinical pathology. Therefore, they remain subclinical for a long time and any method or strategy to detect them might allow chemopreventive therapy. Biomarkers (LOH, genomic instability, DNA aneuploidy, oncogene mutations, overexpression) can be considered signposts that significant tissue damage has already occurred and are potential predictors for cancer occurrence. If their potential can be validated by prospective studies, they will become useful in identifying individuals at high risk of cancer who can benefit from chemoprevention therapies. Nearly all of the genetic, cellular, and circulating markers described in this book are potential intermediate end points of malignancy and should be regarded as potentially relevant biomarkers.

### **Cancer Can Be Cured, But the Effects on Mortality of Present Therapies Have Been Relatively Minor**

Considering that cancer usually starts as a local disease, surgical excision of the primary tumors can cure or, at least, improve quality of life. Over the last few decades, surgeons have abandoned the most mutilating types of surgery in favor of more conservative approaches, often in addition to several other methods of treatment, such as irradiation and chemotherapy. Irradiation should be regarded as essentially a local treatment that can reduce the extent of many cancers, bring some relief from the symptoms, and prolong the patient's life. Chemotherapy is the most widely used form of systemic therapy, but not the only one, for endocrine therapies and immunologic therapies should be regarded as systemic. Despite advances in the treatment of some rare cancers (e.g., germ cell tumors, HD, and childhood tumors), the available methods of treatment are not very successful for the advanced common solid tumors: only about one third of patients survive >5 yr from the time of diagnosis.

In 1987, the first clinical article on the use of a recombinant human (rHu) hematopoietic growth factor and cancer was published (190). In the ensuing few years the introduction of these agents to speed up bone marrow recovery after chemotherapy, and the advent of peripheral blood stem cells (PBSCs), brought hope into the therapeutic arena by allowing a series of clinical studies on the role of intensive and/or myeloablative chemotherapy (191). Recognition that the use of dose-intensive therapies in the setting of relatively resistant hematologic disease resulted in cure rates (in relapsed acute myeloid leukemia) suggested the possibility of applying a similar strategy to some chemosensitive solid tumors (192,193).

In general, in solid tumors, high-dose chemotherapy cannot overcome resistant malignant clones, and only some patients responsive to standard therapy can significantly improve their overall or disease-free survival with high-dose chemotherapy.

Reports from the European Bone Marrow Transplantation trial showed a dramatic increase in overall numbers of treated cases, mainly in patients with lymphoma and breast cancer with poor prognostic features, from 1990 to 1998. This increase was due to the substantial reduction in iatrogenic morbidity and mortality associated with growth factor use, mobilized PBSCs for hematopoietic rescue, and the publication of some encouraging phase 2 results in the treatment of high-risk or metastatic breast cancers.

Fifteen years ago, metastatic breast cancer was invariably regarded as an incurable disease, and patients were treated only with palliative intent. Although the first published attempt to increase the dose intensity of anthracycline-based chemotherapy with rHu granulocyte colony-stimulating factor (rHuG-CSF) in patients with breast cancer was published in 1989 and clearly showed increased objective response rates without significantly increased disease-free survival (194), more myeloablative regimens were studied in this disease with encouraging results by several important groups with the use of bone marrow or PBSC rescue (195). After controlling for known prognostic factors in 1509 North American women with metastatic breast cancer, in a non-randomized analysis of two large, independent data sets, women receiving high-dose chemotherapy had a short-term probability of survival similar to that of women receiving standard-dose chemotherapy but had a modestly higher long-term probability of survival (196).

One reason for the disappointing results for high-dose chemotherapy in breast cancers is related to the great biologic heterogeneity of breast cancers. This relationship is partly illustrated by the molecular diversity of breast cancers, which has led to the identification of high-risk patients by gene expression profiling (197). Moreover, integration of cDNA and CGH microarray data has enabled researchers at the National Human Genome Research Institute in Bethesda, MD, to identify 288 genes whose overexpression in breast cancer is caused by DNA amplification (198). Only a few of these overexpressed genes have been previously correlated with oncogenic function (*myc*, *HER-2*, and *EGFR*). The function of the proteins coded for by most of these genes remains speculative.

The biologic diversity of breast cancers is also illustrated by the variability in growth rates as measured with labeling indices (199,200). Approximately 60% of patients studied had breast tumors with labeling indices <4% and the remaining ranged from 4 to 41%.

The study of nonproliferating cells by kinetic methods remains difficult: the distinction between slowly proliferating and nonproliferating is largely a matter of definition. Effective tumor stem cells are those that under normal growth conditions produce a large number of descendants. Within the tumor, they are usually expected to be undergoing some degree of active proliferation. The larger category, potential tumor stem cells (201), includes not only the former category, but also those stem cells that can be stimulated to proliferate in vivo. After treatment with hormones or cytotoxic agents, some tumor cells will be killed. Among the survivors may be cells that were destined to die or differentiate in the normal process of cell turnover within the tumor but that, as a result of an improved nutrient situation, may act as stem cells for regrowth. In any tumor, a large category of cells is neoplastic in the sense that they have derived from proliferating tumor cells but have irreversibly lost the ability to divide. All cellular kinetic considerations should be remembered when studying the effects of targeted therapies and signal transduction inhibitors. More research is needed to understand the effects of these new types of drugs on tumor kinetics.

Until now, the main and most successful use of growth factors in cancer therapy has not been their direct or indirect antitumor effects, but their use as adjuncts to chemotherapy. Three factors have been registered for clinical application: the myeloid growth factors, rHuG-CSF and rHu granulocyte-macrophage colony-stimulating factor; and erythropoietin (rHuEPO), the main regulator of erythroid growth. The gene for thrombopoietin (also named megakaryocyte growth and development factor) was cloned, and early clinical trials (to determine safety and active dose and schedule) were reasonably encouraging, but no clinical use has yet been found for these recombinant products. Other growth factors are being studied in the clinic (202).

CSFs have been used to support both standard and intensified doses of chemotherapy for almost a decade. Guidelines on their optimal use were initially proposed in 1994 by ASCO (203), and an early European Organization for Research on the Treatment of Cancer (EORTC) report in 1997 (204), among others. The main established indications in cancer medicine (205) are the primary and secondary prevention of febrile neutropenia associated with chemotherapy for the treatment of chemosensitive solid tumors or lymphomas, and the mobilization of PBSCs in the context of high-dose chemotherapy protocols. Reductions in the degree, incidence, or duration of neutropenia are not considered sufficient criteria to justify the clinical use of a recombinant growth factor. Clinically relevant outcomes, such as incidence and severity of febrile neutropenic events and improved quality of life and survival are currently the primary therapeutic end points of cytokine administration.

The discovery that CSF mobilized hematopoietic progenitors from the bone marrow into the peripheral blood was not expected from the early *in vitro* and preclinical work. These cells are now routinely harvested at most cancer centers by one or more apheresis procedures and are usually cryopreserved to be infused after high-dose therapy. Indeed, many of the apheresis devices that were originally developed for plasmapheresis or for the separation of lymphokine-activated killer (LAK) cells were rapidly converted to PBSC procedures. The main advantages of these technologies are a faster trilineage recovery of hematopoiesis after myeloablation (or severe myelosuppression), a reduced requirement for platelet and red blood cell (RBC) transfusions, relative simplicity, and the lack of general anesthetic for harvesting. Although the precise biologic mechanisms that allow PBSC mobilization are still poorly understood, most patients can have PBSCs mobilized by either CSF alone (commonly an outpatient procedure), or chemotherapy followed by CSF (with the advantages of reducing tumor burden *in vivo*). The CD34 assay has proven to be a more reproducible and much faster assay than quantitating the number of GM colony-forming units.

Cellular transfusion therapies began with mature blood cells, first with whole blood and then gradually evolving to fractionated blood cells, proving very effective for RBCs and for platelets, but not for neutrophils because of their kinetics. The second phase of cellular therapeutics began with BMT; a third phase probably started with the advent of recombinant human cytokines (interleukin-2) and LAK cells. A fourth phase (206) has begun with *ex vivo* stem cell expansion technologies (which allow faster hematopoietic recovery or purging of contaminating tumor cells), and *ex vivo* genetic modification of blood cells (in gene-marking experiments), immune cells (to control the severity of graft-versus-host disease after allogeneic BMT by inserting a suicide gene into the T-lymphocytes of the donor), or tumor cells (to stimulate an antigenic response). Gene transfection systems linked with stem cell separation devices or bioreactors, boosting the transfection efficiency of a gene transfer system, are being developed by several

biotechnology companies and may lead to novel forms of anticancer therapy, independent of (or complementary to) traditional chemotherapy.

Antibodies directed against epitopes of epithelial cells often are used to detect minimal numbers of contaminating epithelial tumor cells in bone marrow of patients with solid tumors. The presence of these epithelial cells indicates systemic disease, and for several common cancers (such as breast, gastric, colorectal, and lung), their presence has been reported to be a predictor of distant relapse. Novel approaches, such as RNA-based methods, are less reliable, but techniques such as sequential analysis of gene expression appear promising (207).

Finally, new forms of rHuG-CSF and erythropoietic proteins are entering the clinic (208–210). They tend to have different pharmacokinetic and pharmacodynamic properties that allow less frequent administration of the drug, with comparable efficacy and safety profiles.

### The Era of Targeted Therapies

Arsenicals, which can inhibit at a molecular level by catalyzing the metabolism of *p*-aminobenzoic acid, have recently returned to the clinic, after reports of clinical and hematologic responses by arsenic trioxide in patients with *de novo* and relapsed acute promyelocytic leukemia (APL) (211).

The development of sulfonamides led to the principle of metabolite analogy (i.e., an analog of a metabolite can inhibit a relevant enzyme in a selective and therapeutically relevant manner) and to the hope that the full development of molecular understanding of the action of a drug should mean that the activity of a given structure can be accurately predicted from physical and chemical parameters of that structure. Classic biochemical targets for drug action have included the following:

- Inhibition at the active center (by substrate competition or cofactor inhibition).
- Allosteric inhibition and false feedback inhibitors.
- Other inhibitors acting outside the active center (exoinhibitors).
- Double blockade (many of the disadvantages of structural analogs as simple competitive inhibitors can be overcome if pairs of compounds are used together to block a single biosynthetic pathway).

The discovery of penicillin followed the development of screening techniques that led to the empirical discovery of most antibiotics—toxic ones by the hundred, selectively toxic ones rarely but significantly (212). Cancer cells, of course, pose more formidable problems than bacterial cells. Their complexity is several orders of magnitude higher and they are not foreign cells (like bacteria or fungi), but daughters or granddaughters of normal body cells. Our progress in knowledge will inevitably translate into rapid new therapeutic developments, and, perhaps soon, we shall witness a therapeutic revolution similar to that experienced in the fight against microbial infection after World War II. Scientific discoveries and progress do not follow a continuous or uniform pace but are frequently exposed to bouts of more rapid, discontinuous development. At present, the prospects for this to happen in the fight against cancer seem rather high.

The activity of many of the cytotoxic agents used in today's practice was first detected in the traditional screening methods such as the murine leukemias (L1210 and P388) and murine solid tumors. These cytotoxic drugs were designed to interfere with DNA or chromosomal replication because we were ignorant of the molecular basis of cancer. Enough information is available about the precise molecular mecha-

nisms involved in the regulation of cell behavior (benign or malignant), and soon all the players (regulatory proteins) will be classified into processes (diseased regulatory pathways). It will be possible not only to know the key players in each pathway, but also how each pathway is organized (from upstream to downstream), and where we should intervene therapeutically to correct the malignant behavior of tumor cells (inhibiting angiogenesis, cell growth, or the metastatic process), or to eradicate malignant cell clones (by facilitating senescence and/or apoptosis, or by stimulating the destruction of tumor cells by the immune system) (Table 1).

We can hope to reduce the number of potential therapeutic targets from several hundred to 20 or 30. Most, if not all, of these processes will have upstream regulatory elements (membrane bound or membrane associated), intermediate elements (cytosolic or bound to organelles), and down stream elements (transcription factors or DNA-specific or RNA-specific sequences). New cancer therapies will not be directed to hit the DNA synthetic machinery, but to hit selective targets down-stream from abnormally activated catalytic functions (abrogating the abnormal stimulatory signals), or upstream from inactivated or missing catalytic functions (switching on parallel or alternative regulatory pathways). In simplistic terms, the new therapeutic drugs should block or inhibit activated protooncogenes in their inactive state or somehow lock tumor suppressor proteins in their active state, which is the one normally responsible for inhibiting entry into S-phase or mitosis, or maintaining the normal differentiation and apoptotic pathways (Fig. 3). Because regulatory proteins are usually present in normal cells in either an active form or an inactive form and the chemical equilibrium between the two forms depends on the regulatory microenvironment of the cell, new targeted therapies should be able to correct or at least limit the malignant phenotype by restoring the normal balance between stimulatory and inhibitory signals within the cell.

This concept of regulatory pathway involvement in cell behavior is the basis of a new biochemistry. Crucial alterations in this new biochemistry, perhaps as few as five distinct key molecular changes (213), may be enough to transform a normal cell into a cancer cell. It appears that because of their greater complexity, human cells are more difficult to transform than rodent cells. Simpler organisms, such as fruit flies, very rarely develop malignant cells, despite their ability to develop mutations in an experimental system. No clear explanation has been given for this apparent paradox.

Classic biochemistry taught us how a cell obtains the energy it requires to survive. In 1941, Lipmann postulated that ATP functions in a cyclic manner as a carrier of chemical energy from the degradative or catabolic reactions of metabolism, which yield chemical energy, to the cellular processes, which require energy input. The new biochemistry will tell us how a cell knows what the rest of the cells (in the same tissue or in the rest of the body) want it to do in terms of differentiating (or eventually dying), proliferating, or relocating.

Phosphate atoms are involved in this new biochemistry, for protein phosphorylation is the common result of many signal pathways. In 1937, Cori and Cori began their studies of oxidative phosphorylation. It was not until 1988 that Tonks obtained the first partial sequence of a tyrosine phosphatase. Today, it has been suggested that the human genome might contain as many as 2000 kinase genes and 1000 phosphatase genes (214).

A normal cell under normal circumstances probably decides nothing by itself but merely obeys orders. Normal cells can signal each other in many ways: through pores

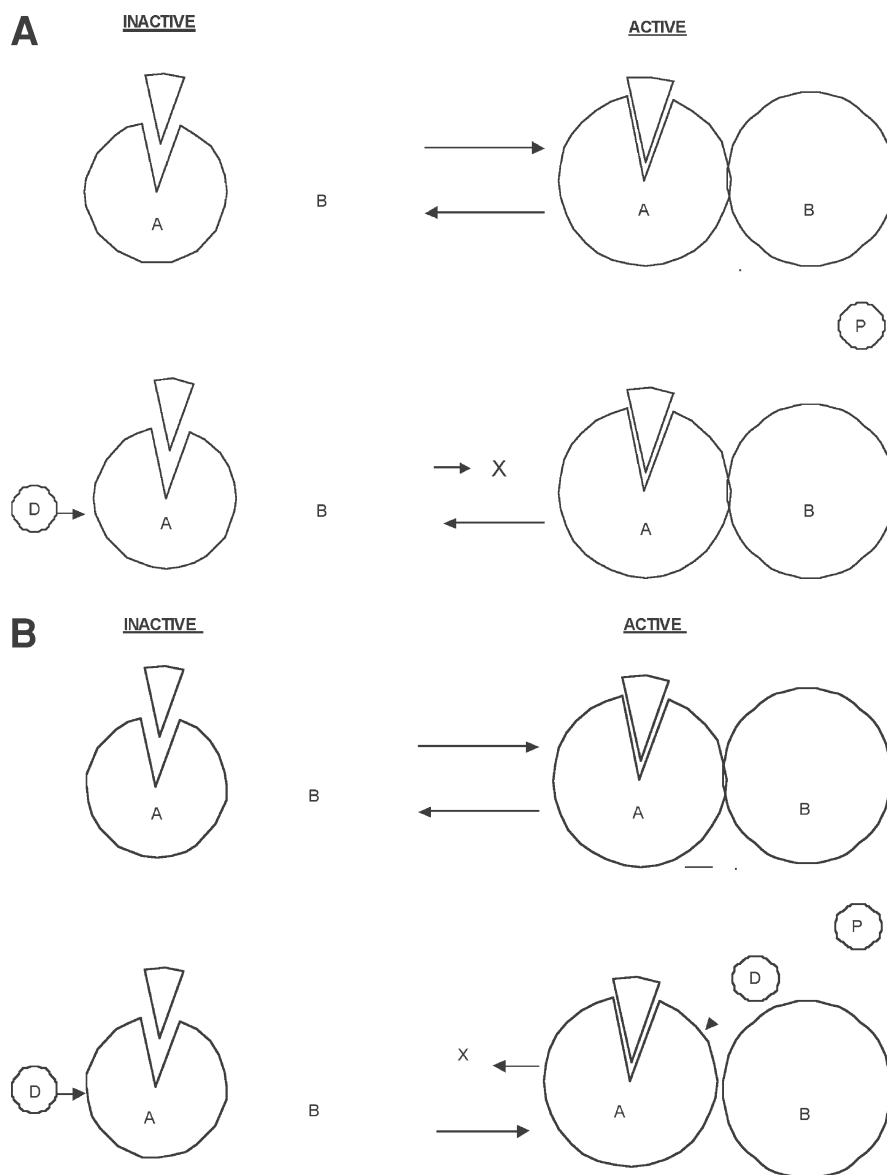


Fig. 3. (A) Oncogenes as targets. (B) Tumor suppressors as targets. A and B, subunits of regulatory molecules; D, blocker drug; P, phosphorylation; triangle, ligand. (See explanation in text.)

in the membranes (gap junctions or plasmodesmata) with specific membrane receptors that recognize soluble or bound ligands (endocrine, paracrine, or autocrine mechanisms), or in some special cases (neurons) by synaptic transmission. A cancer cell (because of mutations, amplifications, translocations, or deletions) makes mistakes (misinterpreting the normal orders or even making them up), and it cannot help doing so because its software has gone badly and progressively wrong. Cancer cells do not have the same underlying chemistry as normal cells of the body, which makes them vulnerable to more specific and selective drugs.

Most intracellular messengers are binary: they are either switched on or off. At any one time, a number of off and on signals travel from the cell membrane to the nucleus (and perhaps also backward) (215). These signals are irreversibly altered in cancer cells with the normal balance between the on and off signals altered. To reestablish the normal circuits or to kill the cell by misleading it into apoptosis, we must learn to be clever, understand these key pathways and develop drugs to block them or to activate alternative complementary pathways. Thus, cancer is a disease of regulatory pathways. Magic bullets may not exist to kill all cancer cells. The signal transduction pathways involved in the proliferative response to various growth factors and mitogens are extremely complex and interactive. They do not act as simple linear cascades, and cross talk is an invariable feature. The new biochemistry is essentially the network of sensing and signaling in cell homeostasis.

Several key questions remain:

- How much selectivity can we expect to achieve with new drugs that will manipulate these complex signaling pathways?
- Will these new drugs be cytostatic, or cytotoxic, or both?
- How will these new drugs be obtained: serendipity, rational drug design, high-throughput screening, combinatorial chemistry, antisense technologies, gene vectors, antigrowth factors, or MAbs?
- Will these new drugs imply chronic or acute therapies?
- Will these new drugs have new and unexpected toxicities?
- Will some malignant clones still develop resistance to signal transduction therapy?

Some of these questions can be answered by the available examples of targeted therapies in cancer. Drugs are being developed for the major cellular regulatory pathways and much progress has been achieved since the publication of the first edition of this book (216–218). Even after what might be a perfunctory examination of the various experimental approaches to targeted drug development so far, it becomes clear that to be more efficient in these efforts, a broader, more global view of the cancer problem, as well as some basic principles based on the biology of carcinogenesis, are needed. Some basic guidelines to select the right targets and a sensible strategy to link discovery, translational research, clinical development, regulatory filings, and marketing aspects of drug development are needed. Not only practicing clinicians who need to make the effort to understand these new concepts and wisely apply them to their patients in the clinic, but pharmaceutical companies and administrative regulatory bodies also must face and solve these important challenges.

Molecular targets likely to lead to significant clinical benefits and to increased overall or disease-free survival are expected to be important for the pathogenesis of a particular form of cancer or several different types of cancer.

To be a good marker of disease is not enough. PSA, e.g., is a very good marker for prostate cancer, but it does not seem to play a major role in the carcinogenesis process of this disease. The Food and Drug Administration (FDA) has not required tests to measure molecular cancer markers to show clinical benefit for approval (219). Similarly, gene expression profiling can reveal hundreds of genes upregulated or downregulated in any tumor. A small proportion of these genes may be shown to be of prognostic significance, but this is not proof that they are relevant to the pathogenesis of the disease. It is reasonable to expect that to be important for the pathogenesis of a cancer, a particular molecular target must, if altered by gain or loss of function, confer a certain selective competitive advantage to the cellular clones affected compared with

normal cells. Relevant molecular targets should be key to other malignant features of cell behavior, such as the capacity to invade neighboring tissues and form metastasis, to induce angiogenesis, or to escape from normal immune control.

From what we have learned so far, these new targeted therapeutic molecules may show a more restrictive pattern of anticancer activity than most conventional cytotoxics now used in the clinic. This pattern represents a financial and strategic problem for pharmaceutical companies. If it costs US \$500 million to develop a drug from the bench to the market and it takes 10 yr or so of laboratory and clinical development, while current patents expire after only 20 yr, then new important anticancer drugs may never report adequate financial returns to the companies, thereby reducing their investment in further research. While it is true that more efficient strategies for new drug development are needed and that substantial amounts of money can be saved by pharmaceutical corporations, it is also true that regulatory bodies (such as the FDA or European Medicines Evaluation Agency [EMA]) should allow more valuable drugs to reach patients as fast as possible, without undermining safety, quality-of-life, and cost-effectiveness issues.

Let us briefly consider two recent success stories: imatinib mesylate and trastuzumab. Both agents represent clinically relevant targeted oriented therapies, although these and other compounds (epidermal growth factor receptor [EGFR], tyrosine kinase inhibitors) that target the upstream components of the signaling pathways often also block the activity of other signaling pathways and are indicated for cancers with a specific growth factor receptor (HER-2/c-erbB-2 in the case of trastuzumab) or tyrosine kinase (Abl-tyrosine kinase in the case of imatinib mesylate). The development of compounds that target the downstream signaling pathways that are hyperactivated in a larger number of cancers may be of greater utility.

Imatinib mesylate, also called STI 571 or signal transduction inhibitor 571 (220), is an orally administered protein tyrosine kinase inhibitor that shows selectivity for the Abl protein tyrosine kinase. The drug blocks proliferation and induces apoptosis of Bcr-Abl-expressing CML and acute lymphocytic leukemia cell lines (221). CML is a hematologic stem cell disorder characterized by the Philadelphia (Ph) chromosome. A reciprocal translocation generates the Bcr-Abl fusion protein and is found in 95% of patients with CML and 5% of adults with acute leukemia. Without treatment, patients with CML can survive 2–3 yr until they develop blast crisis and usually rapidly die from infectious or bleeding complications. With the use of cytostatics (busulfan and hydroxyurea) and interferon (IFN- $\alpha$ ), the rate of cytologic and even karyotypic remissions improved, with overall survival reaching on average 4–5 yr. Although imatinib mesylate also antagonizes the activity of c-kit (stem cell factor receptor) and the platelet-derived growth factor (PDGF) receptor, it proved in early clinical studies to be particularly nontoxic.

Proof of efficacy and validation of the target was a relatively easy phenomenon to study, as the median time from the onset of treatment to a hematologic response (normalization of absolute neutrophil counts with adequate platelet counts and no myeloblasts in the peripheral blood and <5% myeloblasts in the bone marrow) was only 1 mo in all three early studies, and the median time to a major cytogenetic response was 3 mo. Although it is too early to determine response durations and survival, they seem likely to be superior to those achieved with previous therapies. Perhaps a little disappointing was the low rate of complete molecular responses (lack of detection of the Bcr-Abl hybrids by PCR) in the short-term follow-up, and the emergence of resistant clones of



malignant cells. The most troubling side effect was edema, which was usually superficial (limited to periorbital or lower extremity) and responsive to diuretic therapy. Permanent discontinuation of imatinib mesylate because of toxicities was needed in <0.5% of patients. In May 2001, imatinib mesylate was granted accelerated approval for three CML indications: blast crisis, accelerated phase, and chronic phase after failure of therapy with IFN- $\alpha$ . A bonus was the finding of activity in metastatic GI stromal tumors that show activation of c-kit (222). Another example of molecular targeting by this drug is the rare condition called dermatofibrosarcoma protuberans. This disease is associated with activation of the PDGF receptor, usually as a result of a chromosomal translocation. A response in a patient with metastatic dermatofibrosarcoma protuberans treated with imatinib mesylate has recently been documented by follow-up studies with PET and MRI (223). Second-generation inhibitors of Abl tyrosine kinase, with picomolar rather than nanomolar biologic potency in vitro, are in development, as is the combined inhibition of Bcr-Abl and downstream signaling pathways (224).

Imatinib mesylate essentially acts to maintain the molecular target in question (the Abl-tyrosine kinase) in its inactive form (Fig. 3A). Drugs that maintain tumor suppressor molecules in their active form (Fig. 3B), thereby inhibiting tumor progression, are not available. It has been known for some 30 yr that the malignant phenotype of a cell can be restored to normal, or at least suppressed, by cell fusion experiments with normal cells, and Harris first postulated the existence of tumor suppressor elements that could act in a dominant or recessive fashion (225–226).

Another success story is that of trastuzumab, a recombinant MAb against HER-2. A growth factor receptor gene, belonging to the family of human EGFRs is amplified in 25–30% of breast cancers and usually associated with aggressive features. After several clinical and pathologic studies, ASCO (219) recommended that *c-erbB-2* overexpression should be evaluated on every primary breast cancer either at the time of diagnosis or at the time of recurrence. The FDA has approved IHC staining for detecting *c-erbB-2* overexpression and fluorescence *in situ* hybridization (FISH) for quantifying *c-erbB-2* amplification. In 2001, Slamon et al. (227) reported an important prospective randomized study on the addition of trastuzumab to first-line chemotherapy in metastatic breast cancer that overexpresses HER-2. This combination was associated with a longer time to disease progression (median: 7.4 vs 4.6 mo); a longer duration of response; and, most significantly, a survival advantage (5 mo in this high-risk, poor prognosis group of patients). The most troubling, but usually controllable, adverse effect of trastuzumab was cardiac dysfunction, a complication that had not been anticipated on the basis of the results of preclinical or early clinical studies.

High levels of *c-erbB-2* expression or *c-erbB-2* amplification must be used (by IHC staining or FISH) to identify patients for whom trastuzumab may be of benefit. Even in this group of patients, only 50% will show objective responses to the combination of trastuzumab plus chemotherapy (30% if chemotherapy alone is used) (219,227). The reasons for this are unclear. Theoretically, trastuzumab, by interfering with signal transduction emanating from HER-2 (again, blocking the oncogenic molecule into an inactive or less active form), should reduce the potential proliferative competitive advantage of cancer cells over normal cells. The additive or synergistic effects with cytotoxic drugs could be due to yet unknown effects of these drugs downstream of the signaling pathway. Other inhibitors of EGFR (inhibitors of EGFR-associated tyrosine kinase activity) such as gefitinib have not shown survival benefit in the treatment of non-small-cell lung cancer when combined with platinum-based

cytotoxics. Other drugs in development for this indication include erlotinib, ABX-EGF, and erbitux. Caution and more research are needed to discover how to better use these new, promising agents. The rational way to determine the comparative merits of this variety of new agents that block EGFR activity is to conduct clinical trials (228).

A curious case of targeted therapies is that of the PML-RAR $\alpha$  hybrid proteins resulting from the characteristic chromosomal translocation t(15;17) associated with APL. An initial report led to the clinical use of all-*trans* retinoic acid (ATRA) to treat this otherwise usually deadly disease (229). Another observation showed that arsenic trioxide might be active in this disease, with or without concomitant ATRA, in as much as approx 20–30% of patients with this form of acute leukemia relapse despite treatment with ATRA and combination chemotherapy (230).

A special case of targeted therapies is that of endocrine therapies for cancer, which actually constitute the first example of targeted therapies. For example, inhibition of estrogen or testosterone action or production can probably be regarded as the first examples of anticancer-targeted therapies. Beatson first observed tumor regression of breast cancer in some patients after oophorectomy in 1896, but it was not until 60 yr later that estradiol receptors were identified after the localization in the immature rat of radioactive estradiol. How hormones influenced growth and behavior of malignant tissues was not known then, and even today many questions remain unanswered. Approximately two thirds of all patients have positive estrogen receptor (ER) tumors, and about half of them respond to endocrine therapy, at least initially. Somewhat similarly, at least 80% of men with prostate cancer initially respond to androgen withdrawal, but the mean treatment response duration in prostate cancer is only about 2 yr. Both types of endocrine therapy have cytostatic and cytotoxic properties for cancer cells. Neither ERs nor ARs have been described as protooncogenes, even if, theoretically, their mutation into activated forms could lead to some competitive advantage to affected cells. In contrast to the large numbers of missense AR mutations that have been found in association with androgen insensitivity syndromes, there do not seem to be many AR mutations that predispose to human prostate cancer, with the exception perhaps of a missense mutation present in the germline of 0.33% of the Finnish population and 1.91% of Finnish men with prostate cancer (231). After endocrine therapy of prostate cancer, a number of AR gene alterations, in particular, amplification of gene copy numbers, have been described (232). These gene amplifications may reflect an adaptation of the cancer cells to castrate amounts of circulating androgens.

Malignant cells have two basic biologic properties that have not been adequately explained, but that probably depend on some key regulatory pathways irreversibly altered in cancer cells: the loss of contact inhibition of cell growth, and the invasive phenotype (233). The former is also known as the loss of density-dependent regulation and although it has something to do with response to exogenous growth factors, the precise mechanisms that freeze the appetite of the cell for division, when a tissue culture reaches confluence, have not been determined. The transforming growth factor- $\beta$  (TGF- $\beta$ ) family of polypeptides and their receptor pathways have been related to these properties for some time (234).

The invasive phenotype is one of the early steps in the sequence that leads from carcinoma *in situ* to invasive cancer, allowing for the emergence of cellular clones that are able to displace their neighbors and spread through the basement membrane into the underlying tissue. Both behavioral changes are probably dependent on the acquisi-

tion of a new battery of genes expressed or, perhaps more likely, the loss of function of key elements in the regulatory pathways that control cell-cycle check points, cell position, or both. A complete molecular explanation of these phenomena is awaited and should be seen as a priority in current cancer research.

At least 16 ways exist to reduce or abolish the function of a gene product: entire gene deletion, loss of the relevant chromosome, partial gene deletion, disruption of gene structure (by a translocation or an inversion), sequence insertion into the gene, inhibition or prevention of transcription, promoter mutation reducing mRNA levels, decrease in mRNA stability, inactivation of donor splice sites (causing read-through into intron), inactivation donor or acceptor splice sites (causing exon to be skipped), activation of cryptic splice sites, introduction of a frameshift in translation, conversion of a codon into a stop codon, replacement of an essential amino acid, prevention of posttranscriptional processing, and prevention of correct cellular localization of product. Mutation of a gene is not the only way to abolish its function: long-range chromatin alterations, abnormal methylation, and/or imprinting are possible. In human neoplasms, *p16* is silenced in at least three ways: homozygous deletion, methylation of the promoter, and point mutation. The first two represent the usual inactivation events in most primary cancers. *p16* is a very common early event in cancer progression and is frequently seen in premalignant lesions (235). The importance of *p16* is probably similar to that of *p53*. Mutations in *p53* have been found in 30% of human tumors and wild-type *p53* has been reported to suppress tumorigenesis and promote apoptosis (236). The *p53* protein is a potent transcription factor (237) and may promote transcription of genes also involved in carcinogenesis and angiogenesis (236–239).

Loss-of-function mutations usually produce recessive phenotypes, so that as long as one allele remains normal, phenotype is not significantly changed. For a limited number of genes, however, a 50% reduction in the dosage of the gene can lead to phenotypic changes through dosage effect. Certain regulatory functions are inherently dosage sensitive: gene products that compete to determine a developmental or metabolic switch, that cooperate in interactions with fixed stoichiometry, or gene products whose function depends on partial or variable occupancy of a receptor or DNA-binding site.

Less frequently, mutations can lead to gain of function, rather than loss of function, resulting in the ability to acquire a new substrate, overexpress the gene product, maintain receptor in “on” position, keep ion channels inappropriately open, produce structurally abnormal multimers or chimeric genes, bind to new DNA sequences, or trap and inactivate important regulatory molecules. If a protein has several catalytic and allosteric domains, destruction or loss of function of only one of these domains can allow others to be inappropriately activated.

It is possible that some carcinogenic events may include both loss of the natural function of the gene product and gain of a function not normally associated with that particular gene product. A truncated protein might be unable to perform the original function of the native protein, but could interact functionally with other regulatory proteins by exposing the remaining protein domains.

An important issue in new drug development is whether to concentrate on abnormal oncoproteins (mutated forms of the regulatory proteins involved in carcinogenesis) or on the normal counterparts. Although some oncoproteins (Ras in pancreatic cancer) are frequently mutated at the same codon for a particular tumor type, many more derive from very large genes (*BRCA1* and *BRCA2*) or relatively large genes (*p53*) with multiple different possible mutations along the gene, which may differ

according to tumor type and epidemiologic reasons (different ethnic group, contact with specific carcinogens).

It could prove more rewarding to concentrate on normal regulatory proteins (at downstream bottlenecks or points of crosstalk) than on mutated oncoproteins. The problem in this case, however, is that inhibition of normal downstream regulatory oncoproteins might prove more toxic than selective inhibition of mutated oncoproteins.

In general, once the relevant oncoprotein is identified and purified, gene cloning allows the production of sufficient quantities to allow the determination of its main molecular mechanisms (catalytic or regulatory) and its three-dimensional structure. Appropriate molecules, developed by empirical methods, such as high-throughput screening or rational drug design, can be tested in vitro and in preclinical models to find activity and toxicity, and to determine pharmacokinetics and pharmacodynamics. More oncology units will be devoted to clinical testing of new drugs, and cancer research is likely to undergo a rapid growth, provided enough resources are made available.

Cancer is more complex than originally thought. Simple solutions are seldom valid to solve complex problems, although it can also be argued that a simple drug such as cisplatin can cure most germinal cell cancers. The future will depend on finding multiple partial solutions to improve the rate of prevention and cure for this deadly disease.

A global consideration of the matrix of targets (Table 1) helps to understand why it is so difficult to reliably and reproducibly link prognosis to changes in a single molecular marker. Many retrospective studies fail to show by multivariate analysis significant clinicopathologic correlations between a single molecular marker and response to treatment or survival. Moreover, different studies often arrive at apparently contradictory results and prospective studies fail to validate clear-cut relationships. This result may be due to a single marker that is not meaningful outside of the context of the full pattern or matrix of markers. Therefore, all relevant molecular abnormalities present in any given tumor should be taken into consideration.

Elucidation of carcinogenic pathways is also in progress and two examples illustrate the point. According to one model, colorectal cancers can be subdivided into two distinct forms: those belonging to the chromosomal instability pathway and those showing microsatellite instability (MSI) (240). The latter are more often associated with right-side colon cancers and have relatively distinct histologic features. One third or fewer of all MSI tumors arise in patients with HNPCC syndrome. *SMAD4* codes for a protein involved as a downstream regulator in the TGF- $\beta$  signal transduction pathway. Loss of *SMAD4* protein, associated with at least 50% of allele loss at chromosome 18q21.1 in colorectal cancers, can lead to escape from normal TGF- $\beta$ -mediated growth control and apoptosis (241). These *SMAD4* mutations/deletions probably occur before chromosomal instability, but after divergence of the MSI pathway. Of course, other important regulatory pathways, such as Ras and p53, often are altered in colorectal carcinogenesis, as illustrated by the Fearon and Vogelstein hypothesis (9).

The second good example of progress in the elucidation of carcinogenic pathways in a given tissue is malignant melanoma (242). The discovery that >25% of melanoma-prone families carry germline mutations in *CDKN2A*, a tumor suppressor gene on chromosome 9p21, led several groups working with animal models to elucidate the pathways involved in the carcinogenesis process of malignant melanomas, epidemiologically related to fair-skinned people overexposed to sunlight or other forms of artificial UV light, particularly in childhood. *CDKN2A* encodes a protein, also named

INK4A, whose product is a cyclin-dependent kinase (CDK) inhibitor that binds to and inhibits the action of CDK4 and CDK6. These kinases drive the cell cycle by phosphorylating the retinoblastoma protein (pRb), thereby allowing progression from G1-phase to S-phase.

By splicing a different first exon, a situation probably unique in the human genome, *CDKN2A* also yields another tumor suppressor protein, p14ARF (p19Arf in mouse). p14ARF works down the p53 pathway that can lead to either growth arrest if DNA damage by UV light can be repaired by the cell or to apoptosis if the DNA damage has been so great that it cannot be expected to be properly repaired. By contrast, INK4A works down the pRb pathway that can trigger entry into S-phase by becoming hyperphosphorylated and releasing the transcription factor family called E2F. In addition, the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway can take part in the carcinogenesis process, as Ras activation by mutation can lead to an abnormal mitogenic signal and trigger proliferation of mutated cells. A combination of genetic hits in these three regulatory pathways is a likely formula for malignant melanoma carcinogenesis in both mice and humans (243–244).

An important question, when using targeted molecular therapies in preclinical and early clinical models is: Why do some tumor models exhibit regressions whereas others simply slow down or arrest their growth or do not respond at all? New pharmacodynamic approaches are needed to discover what molecular changes are being induced in tumor cells by any of the various genomic and proteomic approaches described earlier, and whether these changes show any dose-response variation or are also influenced by combination with other drugs, such as conventional cytotoxics. In this respect, a critical issue is whether the tumor cells in question maintain or have lost, totally or partially, the integrity of their apoptotic pathways.

E2F is a very important cell-cycle regulatory transcription factor family and at least six human *E2F* genes have been described (245). Because of frequent mutations or deletions in the retinoblastoma pathway, cancer cells very often exhibit increased levels of *E2F* activity, which, by promoting and enhancing the transcription of several protein products needed for entry into S-phase, lead to higher proliferative activity and a competitive advantage over normal cells. High levels of *E2F* also can lead to stabilization of the p53 protein, thereby facilitating apoptosis. Some of the research pioneers in the field advocate for the development of both E2F antagonists (to decrease entry into S-phase) and apparently contradictory E2F agonists to facilitate cell death (246). Other researchers are more cautious and argue that even with sustained high levels of *E2F-1*, cancer cells may escape death by altering the apoptotic signal (247). In other words, mutations/deletions in the p53 pathways, so frequent in cancers, may allow in the presence of high intracellular levels of *E2F* increased cellular proliferation without apoptosis.

Another example, already discussed, is the use of antisense oligonucleotides against E6AP (115) or of small peptides to block the activity of E6 (116) to degrade p53 in cervical squamous carcinomas. It is not only a particular molecular target that matters, but the global overall functional state of regulatory pathways in any given cancer, the matrix of targets (117).

If one looks at the results of cancer research from a perspective of 25 yr, one cannot help feeling satisfied with the results of this research effort in terms of progress in knowledge, although still unsatisfied in terms of therapeutic results. Twenty-five yr ago, the genetic nature of malignant transformation was unknown, and almost nothing was known about growth factors, apoptosis, or regulatory pathways of cell division.

Most alkylating agents, corticosteroids, antimetabolites, endocrine therapies, *Vinca* alkaloids, and antitumor antibiotics were used in the clinic, and platinum analogs were in development. Gene therapy, MAbs, the Human Genome Project, DNA chips, combinatorial chemistry, and high-throughput screening did not exist. Researchers working with yeast models, *Drosophila melanogaster*, or *Caenorhabditis elegans* could not have hoped that their work would become very important for oncologists. Twenty-five yr ago, some prestigious scientists confessed that they could find no reproducible molecular differences between normal and malignant cells that could explain the aberrant behavior of cancer cells. Twenty-five yr ago physicians rarely told patients that they had cancer, and if specifically questioned, they routinely lied to protect the patient. Twenty-five yr ago there was hope (irrational but comforting) that one day someone working with exotic plants or fungi or experimenting in the vaccine world would by chance discover a magic bullet against cancer that would be effective against most common types of tumor.

Perhaps, the closest example we have today to a magic bullet is the use of some humanized MAbs in the treatment of lymphomas, but even in the best of cases, these agents are not specific for cancer cells. The targeted therapy with the greatest impact in the management of B-cell lymphomas is the mouse–human chimeric antibody rituximab (248) that reacts with the CD20 molecule that is ubiquitous on benign and malignant B-lymphocytes. As a single agent, rituximab has been associated with response rates of 50–70% in follicular lymphomas and 15% to 50% in small B-cell lymphomas. In combination with standard cytotoxic chemotherapy, it can produce durable response rates in 90–100% of cases. In a randomized trial in elderly patients, the addition of rituximab to CHOP chemotherapy (cyclophosphamide, doxorubicin, vincristine, and prednisolone) improved survival (249). In April 2002, the radiolabeled antibody Y-90 ibritumomab tiuxetan became available for clinical use in the United States (250) and several more MAbs are being developed.

The therapeutic research effort needed in oncology today will be unparalleled in the history of pharmacology. Hundreds of thousands of new drugs will require clinical testing. New drug development will need to integrate molecular knowledge of specific tumors because each cancer could prove to be different. The molecular changes induced by the test drug will need to be monitored in the tumor itself to confirm that the drug produces the changes in cellular signaling expected before testing. Each cancer will be screened for RNA expression, and automatic PCR and sequencing devices or sophisticated protein chips will provide the clinician with a matrix of targets for that particular cancer in that particular patient. Thus, primary chemotherapy (neoadjuvant therapy) will be more commonly used. Even with the cytotoxic drugs currently available, primary chemotherapy has been shown in several important tumor models (breast, bladder, head and neck, and some lung cancers) to have a potentially better therapeutic index than adjuvant chemotherapies (251). It provides locoregional control, and disease-free and overall survival rates similar to those of current adjuvant therapies, it has the advantage of assessing *in vivo* the response to specific chemotherapy agents or regimens, and it improves the possibility of nonmutilating surgeries (breast-conserving surgery).

Several antiangiogenic drugs are in clinical trials, and the variability seen in their activity suggests that their mechanisms in patients might be more complex and unpredictable than in inbred mice. One theoretical advantage of antiangiogenic drugs is that endothelial cells are less likely than tumor cells to become drug resistant, as they

develop from normal tissue, which is genetically more stable than cancer tissue (252). Integration of clinically relevant inhibitors of angiogenesis into cytotoxic programs (as adjuvant long-term therapy after primary surgery or induction chemotherapy) is expected to improve outcomes.

Considering that many of the noncytotoxic anticancer agents will produce a cytostatic effect rather than frank tumor regression, clinical trial design and end points will need to differ from conventional ones (253). We should not forget that reduction in tumor volume remains important both in terms of symptomatic control and in survival. Cure, or at least long-term disease-free survival, remains the main objective of cancer therapy. It will be of relatively little help to end up with hundreds of new treatments that are not toxic but do not significantly increase cure rates or disease-free survival. It is well known that even with well-conducted phase 3 clinical trials, a single positive result has a definite possibility of being falsely positive, and it generally requires confirmation (254).

Weak drugs on the other hand, might, have some important role in chemoprevention because their use to prevent or delay cancers will need to be protracted in time and cannot be associated with significant toxicities. For common clinically diagnosed advanced cancers, we need new rational approaches to cancer therapy and several combinations of new active drugs cleverly designed and validated. These new drugs should be potent biologic or chemical agents capable of inducing cell death or at least arresting the growth of malignant cells in a durable fashion.

Combinations of new targeted therapies with conventional cytotoxics, on the other hand, might increase tumor cell killing and survival in cases in which the particular combination induces both cell-cycle arrest and apoptosis. In some cases, even molecules that target cell-surface receptors, such as HER-2 in the case of trastuzumab, can induce objective response rates, but often without clear evidence of a dose-response relationship. The main predictor of response may be the degree of expression of the target molecule, as well as the intrinsic relevance of this target molecule of the cancer phenotype. Response rate to single-agent trastuzumab in 111 assessable patients with metastatic breast cancer was 35% for patients whose tumors showed a 3+ staining of HER-2 by IHC, compared with 0% for tumors with only 2+ staining (255). This finding suggests an interesting nonlinear relationship between marker expression and clinical response.

The principle of vaccination is based on two key elements of adaptive immunity: specificity and memory (256). Identification of novel tumor-specific or tumor-associated antigens and a better understanding of the cellular regulation of the immune response has led to a reawakening of interest in the topic of anticancer vaccines and MAbs. Gene therapy of cancer has not led to the clinical successes expected by some of the pioneers in the field, but the rationale for it (i.e., the correction of at least some of the key genetic defects leading to a given cancer) remains valid and new trends are being reviewed.

For those of us who see cancer patients dying almost every day and feel the pain associated with such premature loss of life and human suffering, the intensification and coordination of cancer research is an ethical must. Because the kind of ethics we are doing at the beginning of the third millennium must be based on logic, it follows that we must use the methods of logic. As Immanuel Kant said, to help others because one has kindly feelings toward them is of no moral worth; an act has moral worth only insofar as it is done out of a sense of duty. Politicians and managers of the pharmaceutical indus-

try should also feel this sense of duty to the public and devote more resources and intensify cancer research efforts, without dysfunction or duplication.

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# 2

## Clinical Importance of Prognostic Factors

*Moving from Scientifically Interesting to Clinically Useful*

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**Daniel F. Hayes**

### Introduction

The term *prognostic factor*, when used regarding patients with malignancies, has taken on several meanings. In general, a prognostic factor is considered to be useful because its results serve to separate a large heterogeneous population into smaller populations with more precisely predictable outcomes. In theory, if this separation is both reliable and disparate, one can apply therapy more efficiently to the population by exposing those most likely to need and benefit from the therapy while ensuring that the other group avoids needless toxicities.

In essence, the term *tumor marker* has come to describe a variety of molecules or processes that differ from the norm in malignant cells, tissues, or fluids in patients with malignancies. Assessment of these alterations from normal can be used to place patients into categories that are distinguished by different outcomes, either in the absence of specific therapy or after various treatments are applied.

Tumor markers can include changes at the genetic level (e.g., mutations, deletions, or amplifications), the transcriptional level (e.g., over- or underexpression), the translational or posttranslational level (e.g., increased or decreased quantities of protein, or abnormal glycosylation of proteins), and/or the functional level (e.g., histologic description of cellular grade or presence of neovascularization). Each of these can be assessed by one or more assays, which use one or more methods with different reagents. This enormous heterogeneity of approaches is the root of considerable confusion regarding the value, in clinical terms, of a given tumor marker.

The “molecular revolution” is now well into its fourth decade. Yet, in spite of impressive advances in our understanding of the biology of human malignancy, and in the technology of investigating molecular processes, the number of clinically useful products from these advances is disappointing. For example, in 1995, the American Society of Clinical Oncology (ASCO) convened a panel of experts to establish guidelines for the use of tumor markers in colon and breast carcinoma. Although the expert panel reviewed many putative markers (including both tissue-based and circulating markers), its ultimate recommendations were surprisingly sparse (Table 1) (1,2). In its first deliberations, the panel believed that none of the new molecular markers (e.g., erbB-2, p53, cathepsin D) was established in a scientifically rigorous fashion to be reliable and

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**Table 1**  
**ASCO Clinical Practice Guidelines for Use of Tumor Markers in Breast Cancer (Tissue Factors Only)**

Factor	Use	Guideline
Estrogen and progesterone receptors	Predictive factors for endocrine therapy	Measure on every primary breast cancer and on metastatic lesions if results influence treatment planning.
DNA flow cytometrically derived parameters	Prognosis or prediction	Data are insufficient to recommend obtaining results.
c-erbB-2 (HER-2/neu)	Prognosis	Data are insufficient to recommend obtaining results for this use.
	Prediction for trastuzumab, CMF-like regimens, doxorubicin, taxanes, endocrine Rx	c-erbB-2 should be evaluated on every primary breast cancer at the time of diagnosis or at the time of recurrence for use as predictive factor for trastuzumab; the committee could not make recommendations regarding CMF-like definitive regimens. c-erbB-2 may identify patients who particularly benefit from anthracycline-based therapy but should not be used to exclude anthracycline treatment. c-erbB-2 should not be used to prescribe taxane-based therapy or endocrine therapy.
p53	Prognosis or prediction	Data are insufficient to recommend use of p53.
Cathepsin-D	Prognosis	Data are insufficient to recommend use of cathepsin-D.

Modified from ref. 3.

definitive. A recent update, however, reflects some progress in the field, with recognition of erbB-2 (HER2) as a potential marker for sensitivity or resistance to certain standard therapies against breast cancer, and, more important, as a target of specific therapy itself (3).

Why are the ASCO guidelines so conservative? In reviewing the available literature, the panel recognized that the science of clinical tumor marker investigation had been haphazard and relatively chaotic. Too often, studies of tumor markers are more inclined to be “fishing expeditions” with the hope that something interesting will be detected with statistical significance, rather than being prospective, hypothesis-driven investigations. In light of this confusion, several authors of the guidelines separately developed a proposal for a framework in which previously published tumor marker studies might be critically evaluated. The authors also suggested that this framework might be used by investigators to plan future studies in a fashion that leads to more rapid acceptance, or refutation, of a given marker in the clinical arena. Details of this system, designated the Tumor Marker Utility Grading System (TMUGS), have been published elsewhere (4). The present review applies the principles of TMUGS to examples of evaluations of tumor markers in solid tumors, especially breast cancer, although these systems are certainly applicable to other malignancies in general.

### **Importance of Tumor Markers:**

#### **Adjuvant Systemic Therapy of Breast Cancer as a Case Study**

From the 1950s until about 1985, the annual odds of mortality due to breast cancer per 100,000 women increased steadily in the United States and other western countries (Fig. 1). In the mid-1980s, however, age-adjusted breast cancer mortality plateaued for women in the Western world, and, more recently, mortality from breast cancer has taken a rather dramatic decline (5). While screening and early application of local therapy (surgery, radiation) may have contributed to this decline, it is likely that these encouraging statistics are at least in part the result of widespread application of systemic therapy, including endocrine therapy and chemotherapy (5). Indeed, several meta-analyses of worldwide data from prospective randomized clinical trials have confirmed that adjuvant systemic therapy reduces breast cancer recurrence rates by approx 25% and, more important, mortality by approx 15% in the population of women who participated in these trials, without further subgroup analyses (6–8). These studies are not trials of treatment vs no treatment. Rather, they are trials of early treatment of the entire population vs later treatment of only those who have disease recurrence, if and when metastases occur. Because recurrent breast cancer is rarely if ever cured (9), these data illustrate the high stakes in making decisions about adjuvant systemic therapy.

Given this dramatic and life-saving progress, should *all* patients with newly diagnosed breast cancer be treated with *all* available therapy to ensure maximum benefits? Application of systemic therapy to all patients with breast cancer would be inefficient, with most patients being exposed to toxicities of therapy for little or no benefit. One might argue that the toxicities of endocrine therapies, such as oophorectomy or tamoxifen, are sufficiently tolerable that these therapies are acceptable to most if not all women. Tamoxifen is now used as a “chemopreventive” or “chemoprophylactic” to reduce risk of new breast cancers in women at high risk who have never had the disease (10). Tamoxifen, however, does have relatively frequent bothersome side effects (principally postmenopausal symptoms) and, occasionally, life-threatening toxicities (thrombosis, second malignancies). The side effects of chemotherapy are more dra-

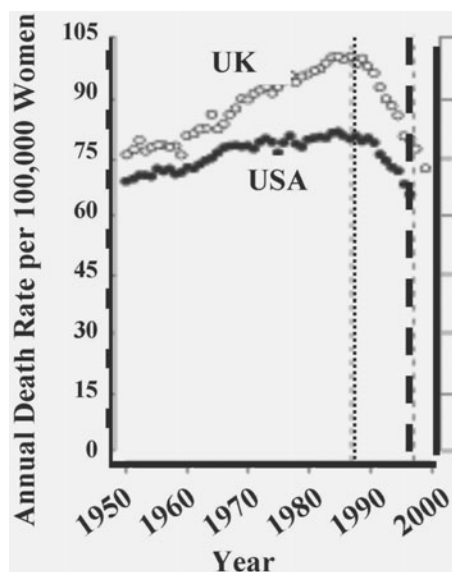


Fig. 1. Annual age-adjusted breast cancer death rate per 100,000 women in United Kingdom and United States: 1950–2000. (Modified from ref. 5 with permission.)

matic, including nausea; vomiting; fatigue; risk of infection and bleeding; and potential long-term complications, such as second malignancies and congestive heart failure.

Factors that might identify those patients most likely to have disease recurrence (designated prognostic factors), and factors that might identify those patients whose disease is most likely to respond to specific therapies (designated predictive factors), would be extraordinarily helpful. However, these factors need to be accurate; if they are not, women who are likely to benefit will be excluded from therapy, blunting the decline in mortality discussed previously. Therefore, a thoughtful discussion of the distinction between these types of factors is warranted.

## Prognosis vs Prediction

Estimating a patient's prognosis requires a complicated set of evaluations, which includes the propensity of a malignancy to expand in volume (proliferative capacity), its ability to escape its natural site of origin and establish growth in a foreign tissue (metastatic potential), and its relative sensitivity or resistance to therapy. Therapies for most solid tumors include surgery, radiation, and/or systemic therapies, such as hormone therapies or chemotherapies. In this regard, the terms *prognostic* and *predictive* have taken on separate meanings (11,12). The prognostic factor designation is usually reserved for those markers that specifically provide an estimate of the odds of a given cancer's recurrence after only local therapy. It is usually a measure of both proliferation and metastatic potential, and it usually implies the odds of systemic recurrence and/or death in a patient who does not receive systemic therapy.

A schematic illustration of a prognostic factor is provided in Fig. 2A. In this case, in the absence of therapy, patients who are positive for the prognostic factor have a worse outcome than those who are negative. Therapy may be effective, but it is equally so (in

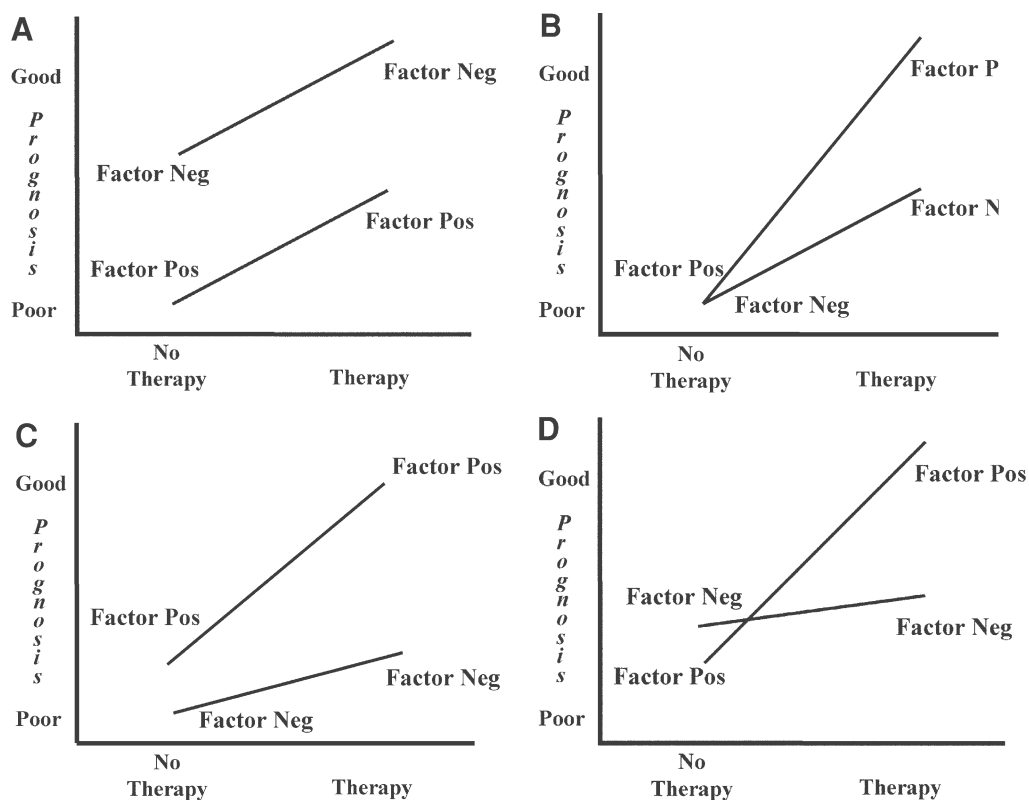


Fig. 2. Schematic representation of prognostic and predictive factors. (A) Pure prognostic factor associated with unfavorable prognosis; (B) pure predictive factor associated with response to specific therapy; (C) mixed factor associated with favorable prognostic and favorable response to therapy; (D) mixed factor associated with unfavorable prognosis but favorable response to therapy. (Modified from ref. 29 with permission.)

relative terms) for both factor-positive and factor-negative patients. The prognosis for factor-negative patients is so favorable that only a few patients, at most, will benefit, even from very effective therapy. Therefore, a prognostic factor is most helpful in determining if a patient is likely to be cured by local therapy alone (surgery and/or radiation therapy), or whether he or she is more likely to have a subsequent recurrence. If so, and if therapy is available that has demonstrated efficacy in that setting, knowledge of an individual's prognosis permits reasonable decision making regarding whether or not application of further therapy is indicated, especially if the therapy is associated with modest-to-severe toxicities. The best examples of prognostic factors for most solid tumors are the Tumor Node Metastasis (TNM) staging systems.

A predictive factor is a tumor marker that helps select therapies most likely to work against a patient's tumor. A predictive factor may be the precise target of the therapy, an associated molecule or pathway that modifies the effectiveness of the therapy, or simply an alteration that is an epiphenomenon linked to the target or pathway of the therapy (such as coamplification of a neighboring gene). A factor that predicts benefit from therapy (a positive predictive factor) is illustrated in Fig. 2B. In this case, the

prognosis in the absence of therapy is the same for factor-negative and factor-positive patients (i.e., it has no prognostic effects). Factor-positive patients, however, have a much better prognosis than factor-negative patients in the presence of the therapy for which the factor is predictive. For example, it is clearly established that estrogen receptor (ER) content in breast cancer tissue is positively related to the odds of response and benefit from antiestrogen hormonal therapy, such as ovarian ablation, tamoxifen, or aromatase inhibitors, because ER plays a fundamental role in estrogen-dependent tumor growth and biology (13). By contrast, *p*-glycoprotein content is a negative predictive factor for resistance to certain drugs, since this protein modulates multidrug resistance by increasing efflux of the antineoplastic agent from the cancer cell (14).

Many factors may be both prognostic and predictive (Fig. 2C). For example, in addition to serving as a predictive factor, ER is also a favorable prognostic factor. Breast cancers with high ER content have generally slower growth potentials, and patients with ER+ tumors have a better prognosis, even if they receive no treatment (15,16).

To complicate this discussion further, some markers may be associated with a *poor* prognosis independent of therapy, but they may predict for an *improved* outcome related to specific treatment modalities (Fig. 2D). One such marker in breast cancer may be the *erbB-2* (HER-2, c-neu) proto-oncogene. Since 1987, conflicting results from several studies have been reported regarding whether *erbB-2* amplification and/or overexpression is a marker of poor prognosis (17–20). *erbB-2* may also be a predictive factor. To add to the confusion, it may be a predictive factor for response to some therapies and resistance to others. For example, *erbB-2* appears to predict relative resistance to hormone therapy and to alkylating agents, but sensitivity to anthracyclines, such as doxorubicin (21). More strikingly, *erbB-2* serves as the target for a humanized monoclonal antibody, trastuzumab. Response and benefit from trastuzumab is closely linked to *erbB-2* amplification and/or overexpression (22,23).

These considerations are often ignored in many prognostic factor studies. Often, a population of patients is studied with a new, putative prognostic factor simply because the samples to be assayed are available and the outcome for the patients is known. Indeed, a prognostic factor can only be evaluated in the absence of systemic therapy, or at least in the absence of any therapy with which it interacts. A predictive factor can only be evaluated in the context of an untreated control group, preferably one that is prospectively identified and followed, as in prospective randomized trials. It is not surprising that studies of a marker that might have both prognostic and predictive capabilities, especially if these effects are in opposition (as may be the case with *erbB-2*) will provide relatively random and conflicting results if not carefully planned with appropriate consideration of treatment effects control groups and satisfactory control groups.

### How Should Tumor Markers Be Selected for Clinical Use?

Ideally, a specific therapy will benefit all those to whom it is administered, and no patient will be exposed to toxicity needlessly. In an imperfect world, however, only a fraction of patients who receive a given treatment will benefit, while all are at risk for the side effects. Although identification of favorable and poor population subgroups is important, simply having a poor prognosis is not justification for treatment. Indeed, many patients will have tumors that are already resistant to specific treatments. In this case, predictive factors will permit selection of those patients who will benefit from the



specific therapy. Unfortunately, treatment for the other patients may not be available or as effective. Therefore, even though their prognosis may be relatively poor, it is unreasonable to expose them to toxicity with no benefit.

Do prognostic and predictive factors exist that permit such elegant selection of patients for treatment? Sadly, in most solid tumors, the answer is no. For patients with newly diagnosed solid malignancies, no prognostic factors predict subsequent recurrence and death with absolute certainty. Therefore, when they are applied in the clinic, both physician and patient must accept some margin of error. These decisions involve a careful assessment of several issues: the degree of separation in outcomes between groups of patients defined by the marker results (marker strength), the reliability of the estimate of this degree of separation (assay methodology and statistical analysis), the magnitude of effectiveness of therapy for the patient's condition (proportional reduction in risk of events), the degree of toxicity of that therapy, and the patient's willingness (as well as the caregiver's and society's) either to forego potential benefit to avoid toxicity or to accept toxicity and cost to gain benefit.

Therefore, part of the art and science of medicine is to determine which markers are most reliable in separating groups of patients who will do well from those who will not, and who will benefit from therapy from those who will not. If done appropriately, tumor marker analysis should permit delivery of therapy as efficiently as possible, providing benefit to the greatest number of patients while avoiding exposure to toxicities as much as possible.

### **Recommending Therapy: How Much Benefit is Needed to Justify Treatment?**

With an estimate of the odds of an event in the absence of therapy (the patient's prognosis), and an understanding of the *proportional* reduction in the odds of an event (such as recurrence or death) due to application of therapy (prediction that a specific therapy will work for a given patient), one can calculate an approximate *absolute* chance of that patient's benefiting from the therapy.

Again, adjuvant therapy for breast cancer provides a useful example. One might estimate, using standard prognostic factors, that in the absence of systemic therapy a patient has a relatively high (e.g., 60%) chance of recurrence and death over the succeeding 10–15 yr after diagnosis. Using predictive factors, one can also estimate the proportional reduction in this chance of recurrence (e.g., 30%) when adjuvant systemic therapy is applied to a population of women with similar characteristics. In this case, a 30% proportional reduction of a 60% absolute risk reduces the odds of an incurable recurrence by 20%. Put another way, 20% of women who would have had recurrent disease if untreated will not as a result of treatment. In this example, the odds of being cured increase from 40% to 60%. Consider another example: the same patient has a favorable prognosis (e.g., a 10% chance of recurrence over 10–15 yr) in the absence of systemic therapy. Applying a similar predictive factor profile, the same therapy will still result in a 30% proportional reduction in events. In this case, however, only 3% of patients will benefit, because 90% will be cured by local therapy alone. In a third example, if the same patient has a 10% chance of recurrence, but the patient's predictive marker profile suggests a 70% proportional reduction in recurrence or death, then the absolute benefit is 7%.

If you were the first patient, would you undergo 3–6 mo of chemotherapy for a 20% improvement in the chances of being alive and disease free for the next 10 yr?

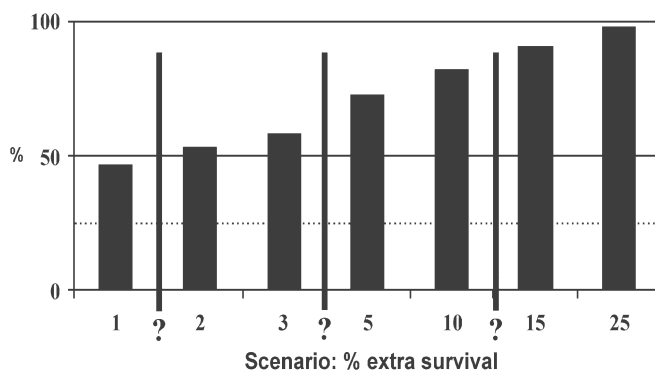


Fig. 3. Fraction of patients with breast cancer who would accept 6 mo of adjuvant chemotherapy according to added survival benefit. Previously treated survivors of breast cancer were queried regarding whether they would be willing to be retreated with 6 mo of adjuvant chemotherapy (cyclophosphamide, methotrexate, 5-fluorouracil) for different scenarios regarding added survival benefits. Question marks represent different cutoffs that might be used to select therapy or not. (Modified from ref. 24 with permission).

If you were the second patient, would you agree to the same therapy for only a 3% improvement in survival? What if you had a favorable prognosis, but your chances of benefit were 7–10%? Several investigators have tried to address this subjective decision-making process with questionnaires that pose these dilemmas to respondents regarding adjuvant therapy for breast cancer (24–26). Such studies are difficult to conduct because an appropriately representative population is not readily identified. Unaffected subjects who are asked to serve as surrogates may not have the same perceptions as they might if truly afflicted with the disease. Patients who must actually decide are often anxious and unsure, and their answers may not reflect their actions. Survivors who are separated in time from the point of making their decision may have considerable cognitive bias, because they may be more willing to accept the therapy that they perceive has led to their current state of well-being. Nonetheless, these studies have demonstrated remarkably similar and striking conclusions. For example, in one study, previously treated survivors were asked if they would reaccept adjuvant chemotherapy (cyclophosphamide, methotrexate, and 5-fluorouracil [CMF]) for 6 mo, placed in the context of various prognostic scenarios (Fig. 3) (24). As expected, most patients stated that they would undergo therapy again when the gains were large (>10% absolute benefit), and a decreasing fraction would be willing to do so as potential gains diminished. More than 50% said they would undergo chemotherapy for gains as small as 3–5%, and nearly 50% would be willing to accept therapy for as little as a 1% absolute improvement in outcome (Fig. 3) (24). Nonetheless, given that a substantial portion of patients would not accept therapy for absolute benefits <10%, accurate assessments of prognosis and prediction are essential.

Similar scenarios can be generated for nearly all medical decision-making situations, assuming that the odds of event occurrence, the proportional odds of reduction of the event, and the toxicities are well established. Indeed, computer models to help breast cancer patients estimate their absolute risks of benefit are now available on the World Wide Web and on CD-ROM (*Adjuvant!* and *Numeracy*) (27,28).

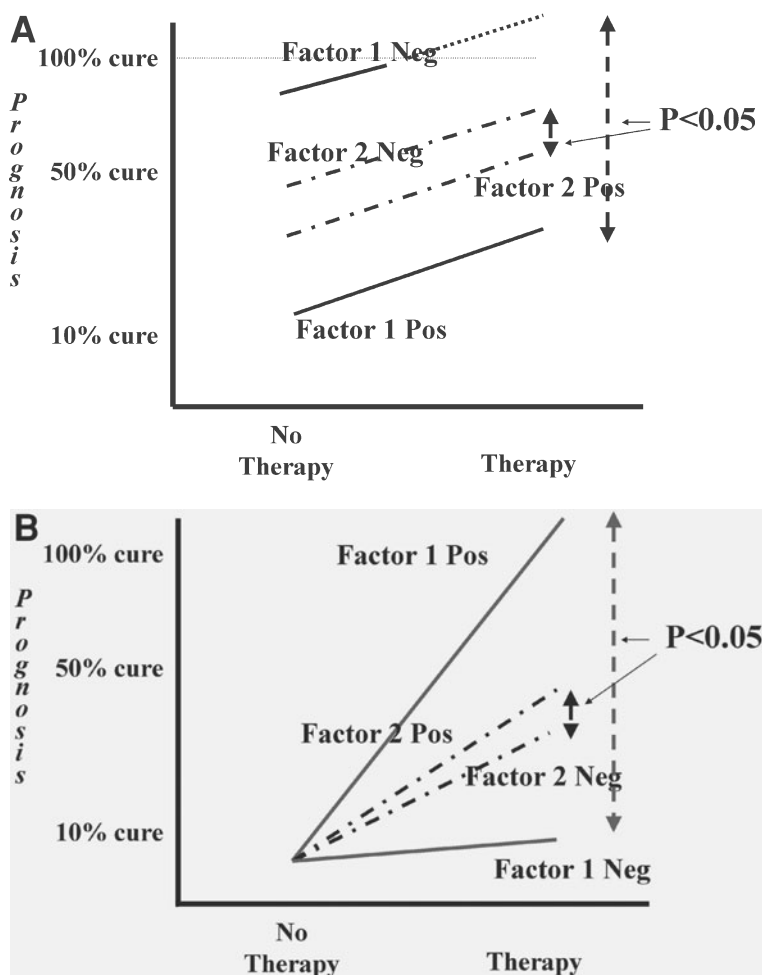


Fig. 4. Relative strengths of prognostic and predictive factors. (A) Relative strength of two pure prognostic factors. (B) Relative strength of two pure predictive factors. Factor 1 (solid line) is a strong factor whereas factor 2 (dashed line) is a relatively weak factor. Both factors reliably separate the population into two distinct groups not owing to chance alone ( $p < 0.05$ ).

### How Can the Relative Strength of a Prognostic Factor Be Determined?

Prognostic and predictive factors can be placed into categories based on their relative strengths to divide a single population into two or more subgroups that have distinct outcomes (29) (Fig. 4A, B). Let us consider two prognostic factors. One factor separates the population very strongly, so that negative-factor patients are very likely to be cured by local therapy alone and positive-factor patients have a very poor prognosis. If effective therapy is available, a sufficient number of factor-positive patients will benefit so that most patients in that population will accept the therapy and its toxicities. The second factor may also reliably separate two groups of patients, with one having a statistically significant more favorable outcome than the other, but not by much. If effective therapy is available, a similar number of patients will ben-

efit in both the negative and positive groups, exceeding the cutoff required for acceptance of therapy as described earlier. Thus, the clinician would most likely use the first factor to help make decisions. Although recognition of the second factor might provide insight into the biology of the disease, it would not have clinical utility.

One can analyze predictive factors similarly (Fig. 4B). A strong predictive factor provides an indication that the therapy is so effective in factor-positive patients and unlikely to be very effective in factor-negative patients that, if the prognosis warrants therapy at all, the two groups of patients would be treated differently. By contrast, the weak predictive factor may provide an indication that factor-positive patients are a little more likely than factor-negative patients to benefit. The *p*-value suggests that these differences are unlikely to be due to chance alone, but the benefit for the factor-negative patients is still sufficient to justify exposure to the therapy.

For patients with newly diagnosed breast cancer, we have proposed three arbitrary categories for both prognostic and predictive factors, based on relative strengths: weak, moderate, and strong (29–31). Let us assume that one can place patients into one of three prognostic categories that fundamentally affects how he/she is treated. Patients with a very good prognosis might not accept any therapy, patients with a modest prognosis might accept some therapy, and those with a poor prognosis would be willing (assuming that effective therapy is available) to accept even more therapy or therapy with more toxicity (Fig. 5). A strong prognostic factor is one that moves a patient across two of these arbitrary prognostic categories, e.g., from very good to poor (Fig. 5). A modestly strong prognostic factor moves a patient less far. A weak factor may improve or worsen a patient's prognosis, but by so little that it is clinically meaningless. These arbitrary categories will differ depending on the disease, the setting, and the investigator/clinician and the patient. Again, using breast cancer as an example, we have proposed that breast cancer prognostic factors that divide the population into subgroups that differ in outcomes (risk of recurrence over 6–10 yr) by two-fold or more are considered *strong* (Fig. 6). Good examples of strong prognostic factors include clinical stage, pathologic identification of axillary lymph nodes, and estimation of tumor size (Fig. 6). Prognostic factors that divide the population into subgroups that differ by 1.5- to 2-fold are considered *moderately strong*. These include tumor grade and perhaps levels of cellular proliferation. *Weak* prognostic factors divide the population into subgroups with outcomes that differ by 1- to 1.5-fold, and include estimates of ER and possibly *erbB-2* content.

Likewise, one can also estimate the relative strengths of predictive factors. The strength of a predictive factor is best determined in the context of a prospective clinical trial in which patients are randomly assigned to the treatment of interest or not. The ratio of the likelihood that a factor-positive patient will benefit from treatment compared with a factor-negative patient has been designated the relative predictive value (RPV) (32). Estimations of the RPV are illustrated in Fig. 6 in which the risk of recurrence for treated patients is compared with the risk for untreated patients for each predictive factor category. As with prognostic factors, three categories of predictive factors have been proposed, based on RPV (Fig. 7). For breast cancer, it is proposed that weak, moderate, and strong predictive factors have RPs of <2-, 2- to 4-, and >4-fold, respectively. The best example of a strong predictive value is ER for tamoxifen, with an RPV >8-fold (7). It also appears that measures of *erbB-2* are very strong predictors for benefit from trastuzumab, although the only available data so far are from patients treated in the metastatic setting (22,23).

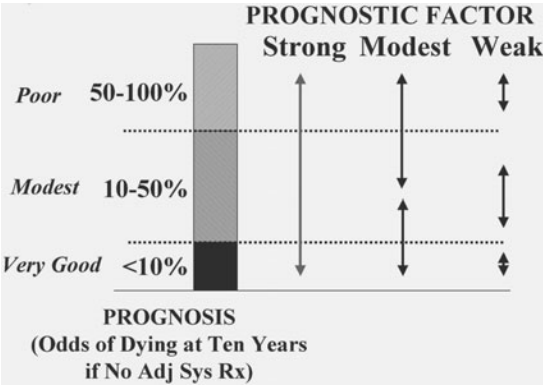


Fig. 5. Schematic model of relative strengths of strong, modest, and weak prognostic factors. A strong prognostic factor moves patients across several prognostic categories. A modest prognostic factor might move a patient across one category, while the weak prognostic factor does not move a patient beyond his or her original prognostic category. Prognostic categories are arbitrarily assigned. (Modified from ref. 30 with permission.)

*If the Relative Risk for Event (Recurrence, Mortality)  
for Positive vs Negative is:*

>2.0	1.5–2.0	1.0–1.5
Accept	Consider	Not Use Routinely

Fig. 6. Arbitrarily proposed categories of relative strengths for prognostic factors (for breast cancer). (Modified from ref. 30 with permission.)

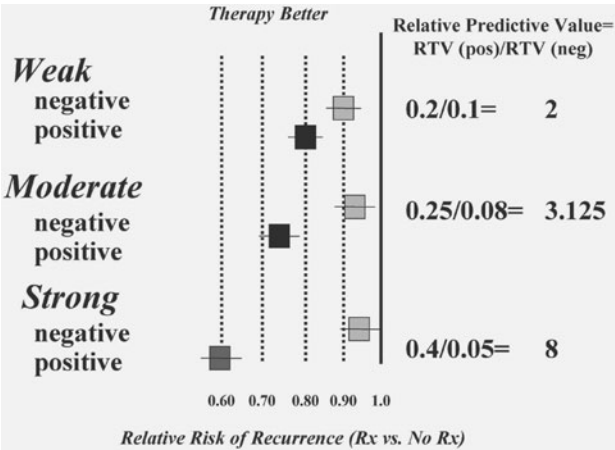


Fig. 7. Schematic model for relative strengths of predictive factors. The relative benefit between groups of patients who are positive (solid bars) or negative (shaded bar) for the predictive factor are indicated. The difference in outcome, charted as proportional reduction in the odds of recurrence for treated vs untreated patients, is relatively small for groups of patients who are separated by a weak predictive factor. This difference becomes larger for those separated by a moderate predictive factor and is quite large for those treated by a strong predictive factor. The solid vertical line (unity) denotes no difference in recurrence between treated patients and untreated patients. (Modified from ref. 31 with permission.)

<i>If the Relative Predictive Value for Event for Positive vs Negative is:</i>		
>4	2–4	1–2
Accept	Consider	Not Use Routinely

Fig. 8. Arbitrarily proposed categories of predictive values strengths for breast cancer. (Modified from ref. 31 with permission.)

An additional concept in this discussion is the issue of residual risk after a selected course of therapy (Fig. 8). If multiple therapies are available, some patients may only need one to achieve a prognosis sufficient to avoid further or more therapy, whereas others may benefit from additional or more aggressive or more toxic approaches. The residual risk is a function of both original prognosis and the relative benefit from specific therapies. A group of patients may have an original prognosis that is sufficiently poor to justify an initial therapeutic regimen. Some may respond so well and benefit so much that their posttreatment prognosis is so favorable that they would elect not to receive more treatment. Other patients might benefit less from the first approach. If further therapy is known to provide additional benefit sufficient to outweigh the risks, then these patients might accept it. Residual risk might be estimated at baseline, before any therapy is given, using initial prognostic and/or predictive factors, as illustrated in the poor prognosis category in Fig. 5. For example, patients with node-positive breast cancer might be willing to accept the increased toxicities of more therapy than patients with node-negative disease. Residual risk might also be assessed at the completion of the initial therapy, however, if markers are available to suggest residual disease burden exists. For example, recent studies of neoadjuvant therapy for breast cancer may permit clinicians to estimate the residual risk for patients after several rounds of chemotherapy (e.g., four cycles of preoperative adjuvant chemotherapy [AC]) based on the presence or absence of residual invasive cancer in the operative specimen (33). Because it has been established that these patients have a relatively poor prognosis, ongoing studies are designed to determine whether these patients benefit from additional chemotherapy.

**How Reliable are the Estimates  
of Relative Strengths of Tumor Markers?**

If clinicians use tumor markers to help patients avoid toxicities of therapy while still optimizing benefit, then they must be relatively confident of the estimates that they have provided to patients. Clinical investigations of new cancer agents are carefully planned, using criteria and terminology that are generally agreed on by most clinical scientists (34). For example, new drugs are sequentially passed through phase 1, 2, and 3 studies, in which toxicity and dose, efficacy, and definitive utility are determined, respectively. In these studies, scales have been developed to describe toxicities, responses, and overall outcomes. Such trials are prospectively planned, with detailed descriptions of the number and types of patients to be studied, how they will be treated, and how the statistical analysis will be performed. Indeed, these rules have been established so that the results of clinical studies approach the same veracity as those from laboratory investigations, in which variables and proper controls can be

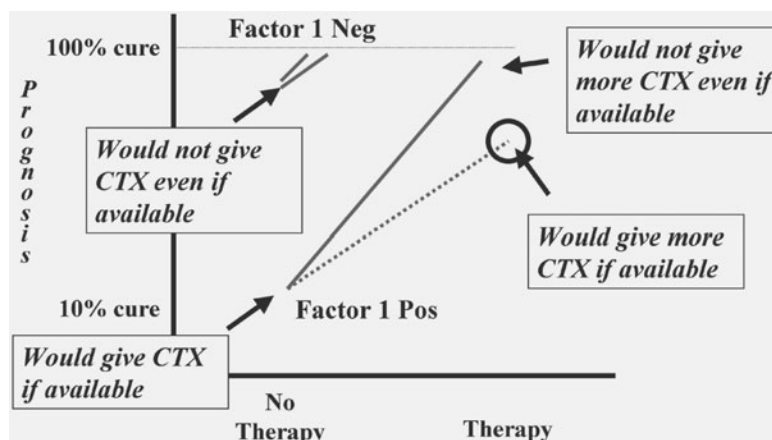


Fig. 9. Schematic illustration of residual risk. See text and Figs. 2 and 4 for details of graphic. Solid lines, patients with little residual risk; dashed line, patients with substantial residual risk.

rigorously defined. Clinical studies that are not so rigorously defined, such as retrospective reviews of clinical experiences, may help generate hypotheses but are rarely accepted as definitive.

In the past, no such consensus system has existed to study tumor markers. More commonly, marker studies are performed using retrospectively available samples from patients treated in a nonuniform manner. Hypotheses are often generated after the data are analyzed, and then presented as fact. Even when multiple studies of the same hypothesis are performed, the populations studied are often heterogeneous and the methods often vary among investigators. Furthermore, negative results are usually not submitted for publication (unless to refute the results of a competing laboratory). It is not surprising that most tumor markers proceed through a typical life cycle before the true utility is accepted or discarded (Fig. 9). In fact, progression through such a life cycle is also common for new therapeutic ideas as well, but because the rules are better established, the time required to reach consensus may be considerably shorter.

We now return to the original TMUGS proposal (35). Determination of relative strengths is only as good as the studies in which they are analyzed. In this regard, the relative quality of the studies is essential in reaching consensus about the strength of the marker. TMUGS was proposed to shorten the life cycle of tumor marker analysis. One component of TMUGS is the importance of a precise description of the tumor marker and the assays used to detect it. A semiquantitative scale, which ranges from 0 to 3+, was incorporated to grade the clinical utility of a tumor marker for any specific use (Table 2). For example, to assess whether a marker should be used to determine prognosis, users are urged to assign a score based on their interpretation of the available published data. A grade of 0 implies that sufficient data exist to conclude that the marker has no utility for that use, while a grade of 2+ or 3+ implies either that the marker should be considered or that it absolutely should be used, respectively, in routine clinical practice. More important, users are encouraged to support their evaluation by determining the level of evidence (LOE) on which their decision is based (Table 3). LOE I data are generated either from a prospective, highly-powered study that specifi-

**Table 2**  
**Potential Uses of Tumor Markers**

Determination of risk
Screening
Differential diagnosis
<ul style="list-style-type: none"><li>• Benign vs malignant</li><li>• Known malignant: tissue of origin</li></ul>
Prognosis
Prediction
Monitoring disease course
<ul style="list-style-type: none"><li>• Detection of recurrence in patient free of obvious disease</li><li>• Patient with established recurrence</li></ul>

**Table 3**  
**Scale to Evaluate Utility of Tumor Markers for Favorable Clinical Outcomes**

Utility Scale	Explanation of Scale
0	The marker has been adequately evaluated for a specific use and the data definitively demonstrate it has <i>no utility</i> . The marker should not be ordered for that clinical use.
NA	Data are not available for the marker for that use because the marker has not been studied for that clinical use.
±	Data are suggestive that the marker may correlate with biologic process and/or end point, and preliminary data suggest that use of the marker <i>may</i> contribute to favorable clinical outcome, but more definitive studies are required. Thus, the marker is still considered highly investigational and should not be used for standard clinical practice.
+	Sufficient data are available to demonstrate that the marker correlates with the biologic process and/or end point related to the use, and that the marker’s results might effect favorable clinical outcome for that use. However, the marker is still considered investigational and should not be used for standard clinical practice, for one of three reasons: <ol style="list-style-type: none"><li>1. The marker correlates with another marker or test that has been established to have clinical utility, but the new marker has not been shown to clearly provide any advantage.</li><li>2. The marker may contribute independent information, but it is unclear whether that information provides clinical utility because treatment options have not been shown to change outcome.</li><li>3. Preliminary data for the marker are quite encouraging, but the level of evidence (see below) is lacking to document clinical utility.</li></ol>
++	The marker supplies information not otherwise available from other measures that is helpful to the clinician in decision making for that use, but the marker cannot be used as the sole criterion for decision making. Thus, the marker has clinical utility for that use, and it should be considered standard practice in <i>selected</i> situations.
+++	The marker can be used as the sole criterion for clinical decision making in that use. Thus, the marker has clinical utility for that use, and it should be considered standard practice.

From from ref. 4.



cally addresses the issue of tumor marker utility or from an overview or meta-analysis of studies, each of which provides lower levels of evidence. LOE II data are derived from companion studies in which specimens are collected prospectively as part of a therapeutic clinical trial, with preestablished end points and statistical evaluation for the marker as well as for the therapeutic intervention. Commonly, an early LOE III study will report an extraordinary difference between two groups delineated by a given tumor marker analysis (Fig. 9). Results from subsequent studies are often more inconsistent. Therefore, we have proposed that the relative strength of a marker for clinical utilities should only be determined within the context of LOE I (or at worse II) studies. In these studies, the marker is the primary objective of a well-designed, highly powered, hypothesis-driven, prospective clinical trial, or it is the objective of a statistically rigorous overview of LOE II and/or III studies. Furthermore, the strength of new prognostic or predictive factors can only be estimated by multivariate analytical methods, including preexisting, accepted factors such as TNM staging and histopathology. It is possible that a marker may be quite prognostic or predictive when considered in a univariate fashion, but that it in fact is only reflecting information already achieved through other, established methods. In this case, acceptance of the new marker would occur only if it can be performed more easily, reliably, or less expensively.

Unfortunately, most tumor marker studies are LOE III, in which specimens happen to have been collected for a variety of reasons and are available for testing a given assay. In general, the authors of TMUGS implied that results from LOE I studies are preferred to assign clinical utility to a marker. Use of a system such as TMUGS to rigorously assess the reliability of assessment of the relative strengths of prognostic and predictive factors will substantially strengthen clinicians' confidence as they counsel their patients.

### **How Can the Relative Strengths of Prognostic and Predictive Factors Be Applied Clinically?**

Outside a clinical trial, there is little value in determining that a patient has a poor prognosis unless therapy is available to change that prognosis. Moreover, if the patient or physician is unwilling to give up any benefit, regardless of how small and regardless of the risks, application of tumor markers is unnecessary unless the results are 100% accurate. Likewise, if the patient is unwilling to accept any therapy regardless of how large the benefit or how well tolerated the treatment, there is no point in applying tumor marker data.

In most cases, however, the patient and physician wish to apply therapy relatively efficiently. In this case, if the patient can judge how much benefit he or she is willing to forfeit to avoid toxicities, one can construct a model in which one marker might be used in some situations but not others (35). Again, the example of application of adjuvant systemic therapy to patients with newly diagnosed breast cancer is used (Fig. 10). In this example, it is assumed that patients can be placed into one of the three prognostic categories based on the odds of systemic recurrence and death during the subsequent 10 yr after diagnosis and local treatment in the absence of systemic therapy: very good (<10% chance of recurrence/death); moderate (10–50%); and poor (>50%). It is also assumed that patients would accept tamoxifen for a small benefit (although not for no benefit), but that they would accept chemotherapy for only a 4% or higher absolute benefit. Additionally, it is assumed that ER is a very strong predictive factor, such that tamoxifen proportionally decreases odds of recurrence by 40% in ER-positive women

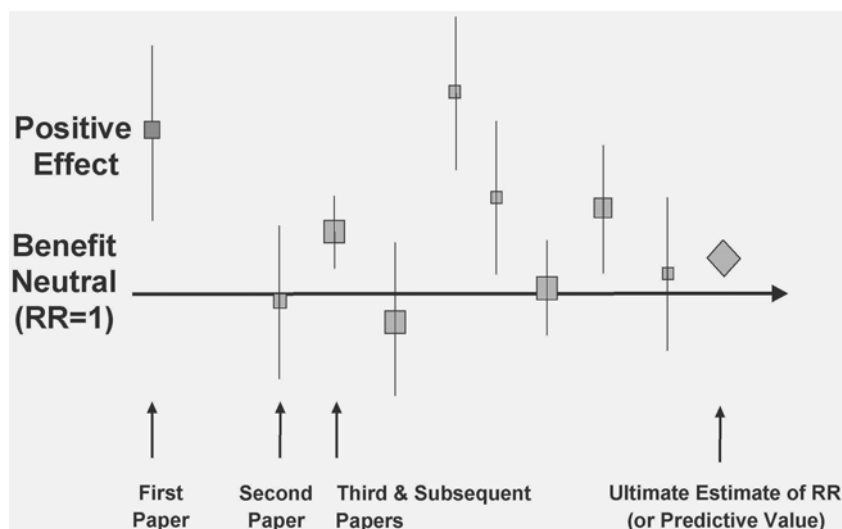


Fig. 10. Hypothetical life cycle of a tumor marker. RR, relative risk.

and not at all in ER-negative patients (7). Finally, it is assumed that different chemotherapy regimens can be applied in sequence with increasing benefits and toxicities, depending on predictive factors.

For example, four cycles of AC might proportionally decrease odds of recurrence by 30% in younger women (<50 yr), but by only 15% in older women (>60 yr) (8). It is also assumed that four additional cycles of a taxane, such as paclitaxel, decrease the odds of recurrence proportionally by a further 20% in ER-negative patients, but perhaps not at all in ER-positive patients (36). (Note that all of these assumptions are approximate estimates based on annual reduction of odds of recurrence calculations.)

Figure 11A provides an example of how the combination of prognostic and predictive factors might be used. The first example might be a postmenopausal 65-yr-old woman with a 1-cm well-differentiated ER-positive breast cancer with no detectable axillary nodal involvement. In the absence of systemic therapy, her overall odds of recurrence over the next 10 yr are  $\leq 10\%$ . Therefore, she has a 90% chance of having been cured by local therapy alone. Tamoxifen will proportionally reduce these chances by nearly 40%. Thus, for every 100 patients who are treated in this situation, 90 cannot benefit because they will not have a recurrence. Of the 10 who were destined to have a recurrence, 3 or 4 will not because of tamoxifen treatment. Because tamoxifen is relatively well tolerated, this percentage of absolute benefit exceeds our cut-off for recommending therapy, and most patients would accept it, resulting in an improvement of their expected cure rate from 90 to approx 93–94%.

Our assumptions suggest that chemotherapy would result in a further 15% proportional reduction in the risk of recurrence over 10 yr for this group of patients. With tamoxifen treatment, this patient now has a residual risk of 6–7% recurrence. A proportional reduction by 15% of this risk represents <1 of 100 patients who might benefit. This is far below the cutoff for acceptability, especially given the toxicities of chemotherapy, and most patients in this group would forgo therapy.

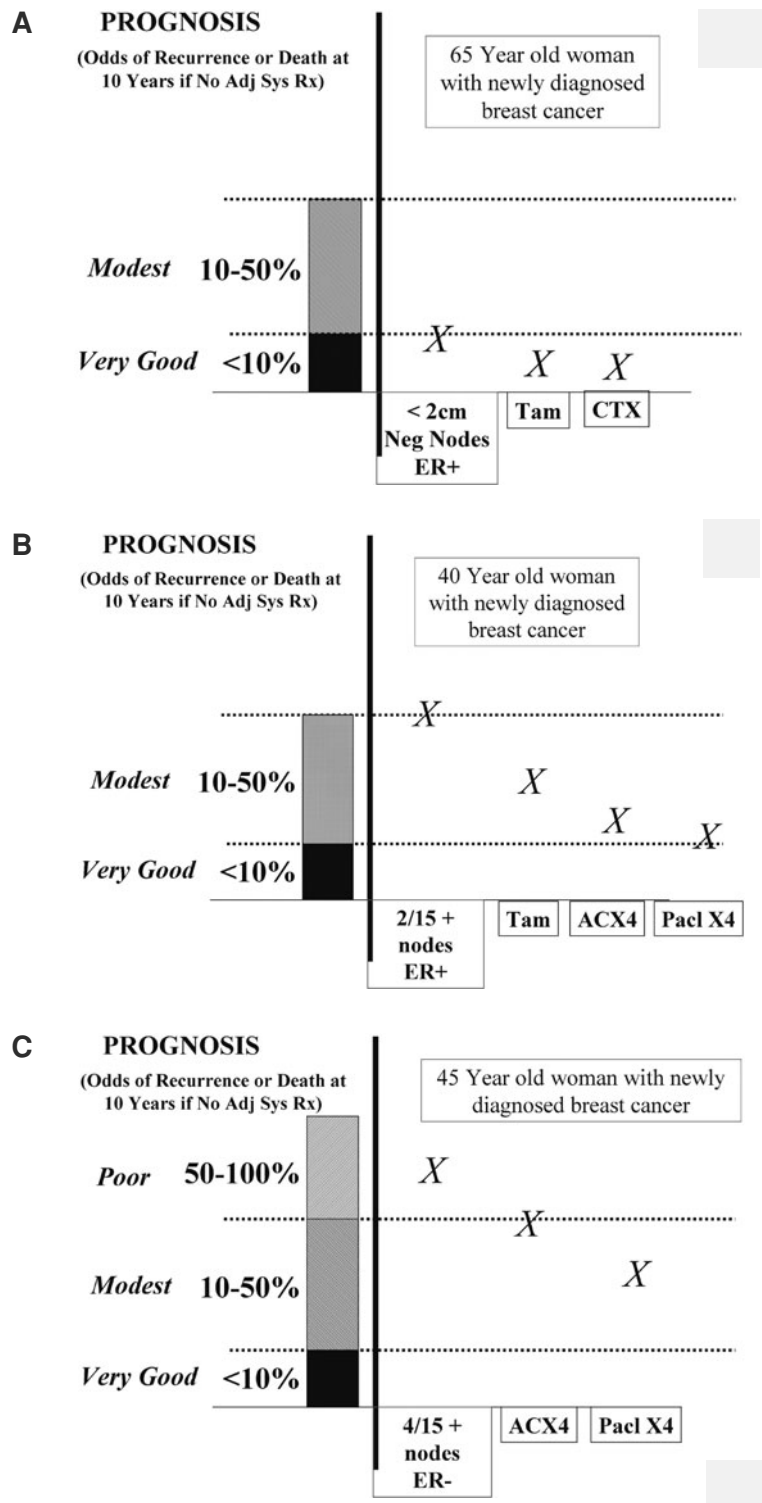


Fig. 11. Schematic illustration of use of prognostic and predictive factors to select appropriate treatments for individual patients with breast cancer (see text for details). (Modified from ref. 42 with permission.)

Consider a 40-yr-old woman with ER-positive breast cancer who has two axillary lymph nodes that are involved with breast cancer (Fig. 11B). In this case, in the absence of systemic therapy, one might estimate that her odds of recurrence over the next 10 yr are approx 50%. As with the first patient, this patient would almost certainly find tamoxifen an acceptable adjuvant therapy. A proportional reduction of 40% would result in approx 15–20 patients who would not have a recurrence, considerably exceeding the cutoff required for recommendation of the strategy. Even if the patient does take tamoxifen, however, her residual risk of recurrence over 10 yr remains approx 20–25%, still in the moderate-risk category. Chemotherapy would result in an approx 30% reduction of this 25% risk, and, therefore, approx five to seven patients would be alive and disease free because of the application of adjuvant AC. It would thus be reasonable to recommend four cycles of AC to this patient.

Should this patient receive four additional cycles of adjuvant paclitaxel? The answer depends on our confidence in the available data. Three prospective randomized clinical trials conducted in the United States have addressed the use of sequential taxanes in this setting. Only one of these has been formally reported, and the results of this trial suggest that the addition of four cycles of a taxane proportionally reduce the odds of recurrence and death by approx 20% (36). An unplanned retrospective subset analysis suggested that this benefit was almost entirely confirmed to the ER-negative subgroup. The results of a second study have only been reported in preliminary fashion. These results suggested no benefit in patients with node-positive breast cancer for an additional four cycles of paclitaxel. No results of the third trial have been reported. It is unclear whether these data, which come from very large prospective randomized clinical trials, should be considered LOE I. Should the clinician wait for more mature data, probably pooled in a “meta-analysis,” before making decisions regarding this extra therapy?

In this example, let us accept the first group’s data. Furthermore, let us assume that *all* patients will have a further proportional reduction and recurrence of 20%, regardless of ER. This patient’s residual risk of recurrence after tamoxifen and four cycles of AC is approx 20% (Fig. 11B). A 20% proportional reduction of a 20% risk would result in a further absolute benefit of an approx 2–4% reduction of recurrence. Does this justify the therapy? This absolute benefit straddles the cutoff to treat or not, and the patient and her physician must discuss this issue carefully.

Finally, consider a 45-yr-old patient with a 4-cm, poorly differentiated ER-negative breast cancer with 4 of 10 involved axillary lymph nodes. This patient’s initial prognosis is poor. In the absence of systemic therapy, one might expect 60–70% of such patients to have disease recurrence within the next 10 yr. Tamoxifen would not be expected to provide any benefit and would not be indicated. Four cycles of AC, with a proportional reduction of 30%, would prevent recurrence in approx 20% of patients, therefore reducing her absolute risk from 70% to approx 50%. Four cycles of paclitaxel would be expected to further reduce her odds of recurrence proportionally by 20%. Therefore, this therapy would benefit approx 10–12 patients. In this case, most clinicians and patients would agree that the additional therapy is indicated.

### **Are There Solid Tumor Markers That Fulfill the TMUGS Criteria for Routine Clinical Use?**

The previous examples regarding breast cancer illustrate how prognostic and predictive markers might be used to tailor patient care in the adjuvant setting. In all malignancies, markers might be used in one of several situations (determination of risk, screening,

**Table 4**  
**Levels of Evidence for Grading Clinical Utility of Tumor Markers**

Level	Type of evidence
I	Evidence from a single high-powered prospective study that is specifically designed to test marker or evidence from meta-analysis and/or overview of Level II or III studies. In the former case, the study must be designed so that therapy and follow-up are dictated by protocol. Ideally, the study is a prospective randomized trial in which diagnostic and/or therapeutic clinical decisions in one group are determined based at least in part on marker results, and diagnostic and/or therapeutic clinical decisions in control group are made independently of marker results. May also include prospective but not randomized trials with marker data and clinical outcome as primary objective.
II	Evidence from study in which marker data are determined in relationship to prospective therapeutic trial that is done to test therapeutic hypothesis but not specifically designed to test marker utility (i.e., marker study is secondary objective of protocol). Specimen collection for marker study and statistical analysis are prospectively determined in protocol as secondary objectives.
III	Evidence from large but retrospective studies from which variable numbers of samples are available or selected. Therapeutic aspects and follow-up of patient population may or may not have been prospectively dictated. Statistical analysis for tumor marker was not dictated prospectively at time of therapeutic trial design.
IV	Evidence from small retrospective studies that do not have prospectively dictated therapy, follow-up, specimen selection, or statistical analysis. May be matched case controls, and so on.
V	Evidence from small pilot studies designed to determine or estimate distribution of marker levels in sample population. May include correlation with other known or investigational markers of outcome, but not designed to determine clinical utility.

Modified from ref. 4.

differential diagnosis, prognosis, prediction, monitoring of disease course) (Table 4) (4). Different markers may perform differently in each situation for each disease (e.g., colon vs breast vs lung cancer). In general, the TNM staging system has been well accepted regarding prognosis for most if not all solid tumors (37). The ASCO Guidelines Panel has made specific recommendations for breast and colon cancer based on data that they believe met criteria consistent with TMUGS (Table 1) (1–3).

Few if any prognostic or predictive factors have been accepted for the other common solid malignancies, such as prostate, lung, and ovarian cancers (38–40). For each, the TNM and grading scales are reliably prognostic. Serial circulating prostate-specific antigen levels and CA125 levels are helpful in monitoring patients with prostate and ovarian cancers, respectively (39,41). For most solid tumors, however, better markers that have been well characterized using results from carefully designed and well-performed studies are urgently needed.

## Conclusion

In summary, the phrase “many are called, few are chosen” seems to reflect the current state of the art regarding tumor marker analysis and solid tumors. The field is evolving rapidly, with a convergence of molecular biology and technology and understanding of clinical trial design and analysis. Several of the large cooperative trialists groups have established separate correlative/biologic committees that are charged with designing hypothesis-driven LOE I and II studies, based on results from pilot studies. The emergence of *erbB-2* in breast cancer as a predictive factor, in a manner similar to ER, may serve as a model of directed studies that lead to determination of the relative strength of the marker, and assignment of a TMUGS score that indicates whether or not it should be used clinically. One hopes that careful and thoughtful consideration of study design will considerably shorten the life cycle required to bring a tumor marker from the laboratory to the clinic.

## Acknowledgments

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# 3

## Genetic Markers in Sporadic Tumors

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Maria Oggionni, Silvana Pilotti, and Marco A. Pierotti**

### Introduction

Progress in understanding the molecular basis of neoplastic transformation has strengthened the concept that cancer is a genetic disease. This concept, however, lumps together two types of genetic diseases with the same outcome: the first linked to an entirely somatic cell–gene deregulation and the second dealing with a genetic susceptibility. At the somatic cell level, deregulation of cancer genes that control the careful balance between increase in cell number and withdrawal from the cell cycle promotes neoplastic growth by disrupting this balance. This deregulation occurs as a result of circumvention of the apoptotic machinery, promotion of cell division and cell proliferation, loss of cell differentiation pathways, and disruption of cell–cell communication and interaction. Thus, cancer represents the end point of a multistep process involving cancer genes and stimulatory and inhibitory signals provided by and controlled by products of the cancer genes.

Moreover, an additional feature of the cancer phenotype is the ability of a cancer cell to modify its environment, including promotion of angiogenesis, degradation of stroma organization, and production of factors related to inflammatory processes.

In the first type of genetic disease, alterations in cancer genes can involve either dominant, gain-of-function mutations within protooncogenes that result in abnormal positive signals for cell proliferation, or recessive, loss-of-function mutations within the tumor suppressor genes (TSGs) that interfere with the negative regulation of cell growth. Mutations within TSGs may have a dominant-negative effect, one in which an altered protein is produced that competes with its wild-type counterpart and prevents its activity. Mutant versions of TSG *TP53* provide an example of such a mechanism. A third type of cancer gene has recently been identified in colorectal tumors associated with hereditary nonpolyposis colorectal cancer (HNPCC). These genes control mismatch repair (MMR), a process associated with the fidelity of DNA replication, and have been designated mutator genes. Their alterations cause microsatellite instability (MSI), characterized by random contractions or expansions in the length of simple sequence repeats or microsatellites, and may have important prognostic implications.

The second type of genetic disease is based on the recognition of a genetic susceptibility in approx 8–10% of patients with cancer. This latter disease results from the

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inheritance of altered alleles of genes, which are almost always of the tumor suppressor type. Along with different penetrance, this tumor suppressor type determines the genetic risk of cancer, which can be almost 100% during a lifetime. In all cases, including those genes that predispose to a genetic risk of cancer (1), alterations of cancer-associated genes define molecular markers. These markers are useful for novel diagnostic approaches and for genetic profiling of the tumor cell, with the aim of providing better prognostic evaluation and prediction of therapeutic drug response.

To provide a more rational view of the problems, this chapter organizes the subject by discussing genetic markers from cancer-associated genes that were altered by point mutation, deletion, or inappropriate expression at the somatic cell level. The classes of genetic markers derived from chromosomal instability and from nonrandom chromosomal abnormalities and generating tumor-specific fusion product are also discussed. Finally, some relevant pathways whose perturbation can lead to neoplastic growth are illustrated.

## Genetic Markers Derived from Cancer-Associated Genes

### TP53

*TP53* is located on chromosome 17p13.1 and encompasses 20 kb of DNA. This gene is composed of 11 exons, the first of which is noncoding. In cross-species comparison, the p53 proteins show five highly (>90%) conserved regions within the amino-acid residues that are considered essential to the functional activity of p53 (2). *TP53* encodes a 393 amino acid nuclear phosphoprotein, which contains phosphorylation sites at the amino (N) and (C) carboxy termini, a central zinc-binding core domain that interacts with DNA, and a nuclear localization and tetramerization domain at the C-terminus.

p53 is expressed at low level during homeostasis. The amount of this protein increases in response to a variety of stress signals including DNA damage as well as arrest of DNA or RNA synthesis, such as ionizing or ultraviolet irradiation and DNA-damaging drugs. Increasing the amount of protein induces the delay of cell-cycle progression from G1- into S-phase, which allows DNA damage repair. If DNA repair is unsuccessful, programmed cell death (apoptosis) occurs. A p53-dependent pathway may also regulate the G2/M-phase transition. Evidence of these effects on cellular growth and death has led to the view of p53 as guardian of the genome (3).

The primary mechanism by which p53 negatively controls the cell cycle is the transcriptional activation of *WAF1/CIP1* gene, the protein product of which, p21, binds to cyclin/cyclin-dependent kinases (CDK) complex and inhibits retinoblastoma protein (Rb) phosphorylation with consequent arrest in the G1/S-phase.

*Gadd45- $\alpha$*  is induced by p53 protein in response to DNA damage. The *Gadd45* gene product interacts with various cell-cycle-related proteins such as Cdc2 and PCNA and contributes to G2/M-phase cell-cycle arrest (4,5). Damaged DNA may be blocked by the G2 checkpoint mechanism before entry into mitosis. Evidence implicates p53 in this type of cell-cycle control. The p53 transcriptional-activated target genes involved in this process include *14-3-3-sigma*, *B99*, and *Reprimo*.

14-3-3-sigma belongs to an emerging family of proteins that bind to Ser/Thr phosphorylated residues. This protein induces cell-cycle arrest in G2 by sequestering nuclear proteins required to enter into mitosis (e.g., Cdc2) in the cytoplasm (6). *Reprimo* is a glycosylated cytoplasmatic protein able to induce cell-cycle arrest by inhibiting Cdc2 activity without interfering with Cdc2/cyclin B1-complex (7). *B99* protein may cause G2 arrest independently of Cdc2 through an unknown pathway. It has been suggested

that B99, interfering with microtubule rearrangements required to enter into mitosis (formation of a spindle), may trigger G2 arrest (5,8). Under extreme stress and severe DNA damage, p53 can also induce activation of genes implicated in the apoptotic cascade, including *bax*, *DR5*, *PIDD*, *NOXA*, *PUMA*, and *Fas/APO-1* (9–13).

It is quite reasonable to speculate that p53-mediated apoptosis is a consequence of the combined expression of the various target genes, the expression of which is modulated in the cell type- and/or cell stress-specific manner. Wild-type p53 is known to repress a number of cellular promoters that may be important for an apoptotic response in the cell-cycle control. For example, expression of the survival factor *bcl-2* is in general suppressed by wild-type p53 and endogenous genes, such as *MAP4* and *Stathmin*, which are both involved in microtubule polymerization and repressed by wild-type p53 (14).

The ability of p53 to exert regulation of such a large subset of genes can be attributed to its selective association with different transcription factors. This association is related to posttranslational modifications of p53. More than 18 phosphoacceptor sites were reported for p53 protein, most of which are modified in response to damage or stress, although several sites are phosphorylated under normal growth conditions (15,16). Phosphorylation of p53 differs during cell-cycle progression in normal growing cells and coincides with the ability of p53 to bind to some of its regulatory proteins, including p300, mdm-2, and JNK (17). The complex phosphorylation of p53 is mediated by multiple kinases, including ATM, ATR, and Chk2 (16,18,19).

In addition to the complex pattern of phosphorylation, acetylation appears to influence p53 activation and function. Different histone acetylases such as CBP/p300 and PCAF are known to modify p53 in several Lys residues in the C-terminus (14,20). Most important, Lys320 and Lys382 become selectively acetylated in vivo in response to DNA damage. It is likely that regulatory mechanisms of phosphorylation in the N-terminus residues of p53 facilitate the acetylation of C-terminus sites, leading to activation cascade in response to DNA damage (21).

*TP53* mutations are involved in almost all tumor types, especially carcinomas of the colorectum, breast, lung, esophagus, stomach, liver, and bladder; the frequency and type of mutation differ substantially among cancers. A database of these mutations is maintained at the International Agency for Research on Cancer (IARC). This database currently contains >15,000 entries, representing the largest set of information on human mutations associated with cancer (22). More than 80–90% of the *TP53* mutations reported for human cancers are clustered between exons 5 and 8 within the evolutionary conserved regions of the gene (codons 110–307). Exons 2 and 11 contain only 0.1% of the 15,000 mutations identified from the IARC *TP53* database. The mutations are usually missense, giving rise to altered proteins. Several sites where mutations are detected at high frequency are referred to as hot-spot mutations, and these include codons 175, 248, 273, and 282. Most of these mutations are present in heterozygosity with the wild-type allele of the gene (3,23). The loss of wild-type p53 tumor suppressor function may occur by dominant-negative inhibition of wild-type p53 function. This effect corresponds to the capacity of the mutant protein to complex with the product of the remaining wild-type allele inactivating its function (24,25).

Although mutations have been extensively discussed as a means of wild-type p53 inactivation, mutation-independent mechanisms can inactivate p53 function. Overexpression of mdm2, which occurs in approx 20% of soft-tissue sarcomas in humans in presence of wild-type *TP53* alleles, inhibits p53 function by targeting p53 for degrada-

tion. Furthermore, *P14<sup>arf</sup>*, a product of *INK4a/CDKN2A* locus, may bind mdm2 and neutralize its capacity to repress p53 (26). Human papillomavirus (HPV) subtypes 16 and 18 express the E6 viral oncoprotein, which may target p53 through an ubiquitin-dependent process (27). The silencing of proapoptotic factors, such as Apaf-1 (28), may lead to the interruption of p53 downstream signaling.

Alterations in *TP53* can be detected though single-strand conformation polymorphism (SSCP), denaturing-gradient gel electrophoresis (DGGE), temperature-gradient gel electrophoresis (TGGE), constant-denaturant gel electrophoresis (CDGE), denaturing high-pressure liquid chromatography (DHPLC), oligonucleotide array assay, yeast functional assay, pyrosequencing, or sequencing analysis.

Recent results suggest that *TP53* mutations may be one of the most important predictors for poor prognosis, increased risk of relapse (29), and cancer death risk (30,31). Unfortunately, the clinical application of *TP53* mutation status as a prognostic tool remains controversial. Apparently, each *TP53* mutation confers different biologic properties that seem to influence the prognostic significance of mutated p53 protein in tumors. *TP53* has potential clinical applications in early detection strategies. Detection of *TP53* mutations in sputum of patients with lung cancer, combined with *K-ras* mutation analysis, may be a suitable target for early detection strategies (32). This method does have a limitation: the percentage of tumor cells identified is very low. In non-small cell lung carcinoma (NSCLC), missense mutations rather than null mutations were reported to be associated with poor prognosis (33). Again in NSCLC, another study showed that the assessment of *TP53-C/Ki/Ras-C/erbB-2* mutational profile may be useful applied to define low- and high-risk groups for treatment failure in patients undergoing potentially curative resection (34).

The potential clinical application for prognosis with *TP53* has been studied. Analysis of *TP53* mutations in fine-needle aspirates (35) or core biopsy may be useful for prognostic assessment and prediction of response to adjuvant chemotherapy before radical surgery in patients with breast cancer. Missense mutations in the L2 and L3 loops from the zinc-binding domain of p53 protein were found to be associated with decreased disease-free and overall survival of the patients (29,36). These types of mutations were associated with resistance to doxorubicin therapy (37). In patients with node-positive breast cancer, detection of *TP53* mutations, combined with the detection of *HER-2/neu* gene amplification (31) in surgical specimens, can be used as a molecular tool for prognostic assessment (29,37) and therapy planning. The most important subgroup of patients with breast cancer for whom reliable prognostic factors are needed is women without axillary lymph node involvement. Introduction of routine *TP53* mutation screening may be useful to assist in the selection of patients with node-negative breast cancer who can be considered for postoperative adjuvant treatment (38,39).

In colorectal cancer, detection of *TP53* point mutations, particularly mutations in the conserved domains (e.g., Arg175) (29) may be a valuable prognostic adjunct in defining more aggressive tumors. Multivariate analysis has shown that survival in colorectal cancer is strongly related to the presence of *TP53* mutations alone, or in combination with *K-ras* mutations (40,41).

*TP53* mutations are frequent in late-stage ovarian cancer (50% of stage III and IV epithelial ovarian cancers) and have been associated with reduced overall survival rates (42). A study confirms previous reported data showing a significant correlation between the presence of *TP53* missense mutations (43) and resistance to cisplatin-based therapy in ovarian cancer (44). Additional findings (45,46) suggest that inacti-

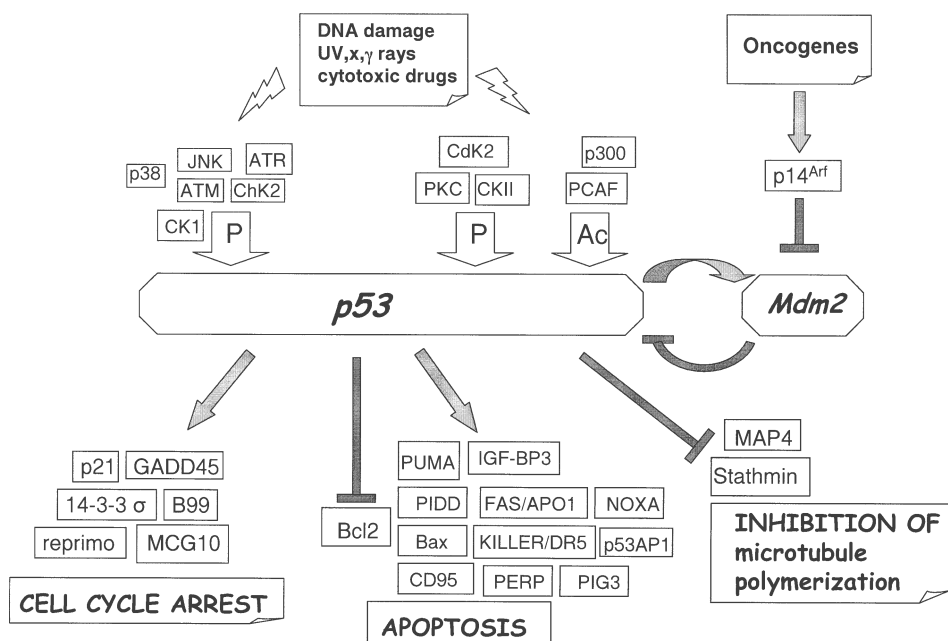


Fig. 1. p53 pathway.

vation of wild-type *TP53* may confer an increased sensitization to the chemotherapeutic agent paclitaxel and help overcome resistance in tumors that do not respond to platinum drug alone. These results could make the detection of *TP53* mutations a useful marker for therapy planning. Conflicting findings imply the need for further studies to elucidate the role of p53 in the response to chemotherapy in ovarian cancer.

In bladder cancer, *TP53* analysis shows that patients harboring a normal p53 pathway had a low death rate and could be considered for a low-risk category. By contrast, patients with an abnormal p53 pathway are found to have an aggressive course of their disease, a high death rate, and could be considered as high-risk cases (47,48).

In head-and-neck cancer, mutations in p53 protein DNA contact domain confer an accelerated tumor progression and reduce therapeutic responsiveness toward adjuvant radiation therapy (49,50) and toward cisplatin–fluorouracil neoadjuvant chemotherapy (51). Taken together, these data suggest that the loss of function of p53 protein and the type of mutation may lead to a more aggressive or more treatment-resistant tumor phenotype. A summarizing scheme of p53 interactions is shown in Fig. 1.

## ras

*ras* genes belong to a multigene family that is highly conserved among eukaryotes, including mammals and yeasts, suggesting that they may play a fundamental role in cellular proliferation. The *ras* genes are named H-*ras* (homologous to Harvey murine sarcoma virus oncogene), K-*ras* (homologous to Kirsten murine sarcoma virus oncogene), and N-*ras* (initially isolated from a neuroblastoma cell line) (52). The human *RAS* genes encode for similar membrane-bound 21-kDa proteins (189 amino acids) involved in signal trasduction, with a guanine nucleotide-binding activity as well as an intrinsic guanosine 5'-triphosphatase (GTPase) activity. Normally, these

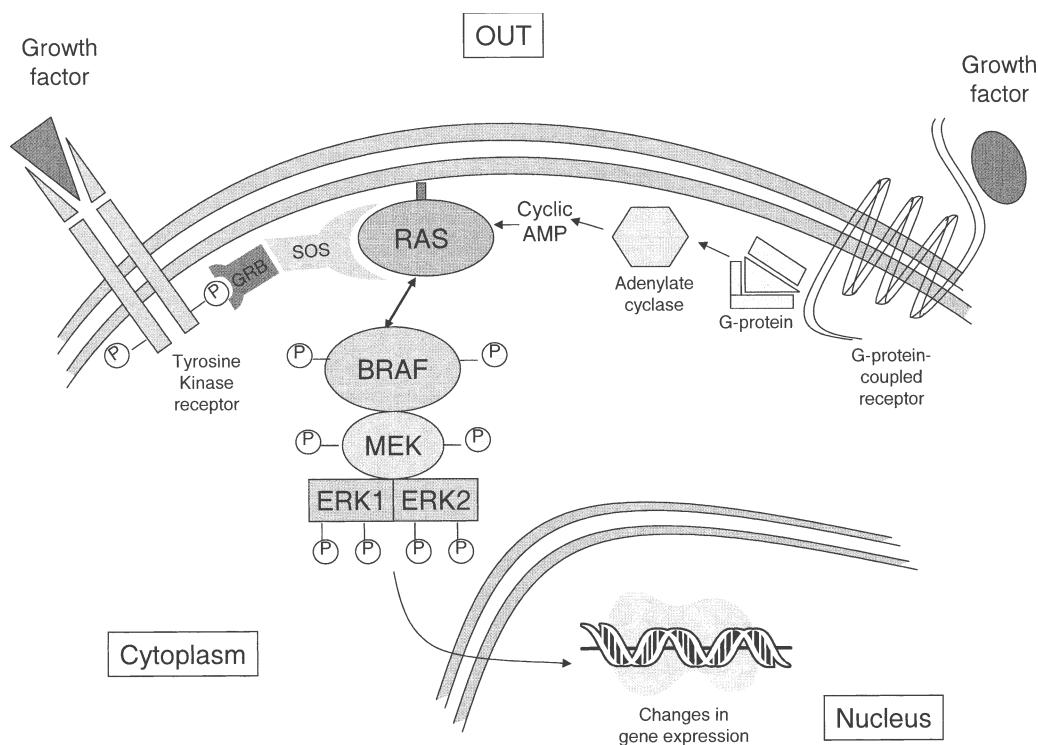


Fig. 2. RAS-RAF-MAPK signaling pathway.

proteins (p21ras) exist in equilibrium between an active and inactive state. The p21ras proteins, characterized by a conformation that allows binding to guanosine 5'-diphosphate (GDP), remain inactive until they receive a stimulus from another protein upstream of the transduction pathway. This stimulus results in the exchange of GDP for guanosine 5'-triphosphate (GTP) followed by conformational change of p21ras to its active state. The activated proteins transduce the signal by linking tyrosine kinases to downstream Ser/Thr kinases, such as raf, and mitogen-activated protein kinases (MAPK) (52). They subsequently become inactivated by their intrinsic GTPase activity, which catalyzes the hydrolysis of GTP and permits the return to the inactive GDP-bound state (53) (Fig. 2).

Stabilization of ras proteins in their active state causes a continuous flow of signal transduction, which results in malignant transformation. p21ras proteins can acquire transforming potential secondary to a point mutation at codon 12, 13, or 61 in the coding gene. Transformation can occur with mutations at or near the GTP-binding domain of p21ras proteins, which prevents the inactivation of GTP and results in continuous p21ras activity (52). Normal *ras* genes can induce malignant transformation if highly overexpressed (53).

*ras* are the most frequently activated oncogenes found in a variety of human cancers, including adenocarcinomas of the pancreas (90%), colon (50%), and lung (30%). Adenocarcinomas show mutations in *K-ras* oncogene, predominantly in codon 12 with a G-T transversion. Methods for detecting *ras* alterations include SSCP, CDGE, DGGE, restriction fragment length polymorphism (RFLP) analysis, allele-specific oligodeoxynucleotide hybridization, and sequencing analysis.

*ras* has potential clinical applications in the early detection of pancreatic tumors. Because 80% of pancreatic carcinomas contain *K-ras* mutations, the detection of *K-ras* mutations in pancreatic juice combined with other somatic mutations common in this cancer (e.g., *TP53*) might be a valuable tool for the detection of pancreatic carcinoma (54,55). *K-ras* molecular analysis, however, can only complement the cytologic evaluation, since *K-ras* mutation is not specific and is present in pancreatic juice of patients with chronic pancreatitis (56,57). Detection of *K-ras* mutations in stool specimens could be a noninvasive presymptomatic indicator of colonic adenomas (mostly adenomas >1 cm) (58) or early colorectal tumors (59). A highly specific molecular detection of *K-ras* mutation in stool has been achieved (60,61). *K-ras* mutations are present only in 50% of colorectal tumors, and successful detection of *K-ras* alterations in stool depends on tumor location (60,62). The detection of other mutant genes (e.g., *APC*, *TP53*), currently expensive and time-consuming, is advisable to increase the potential sensitivity of this strategy (60,63).

As a prognostic tool, evidence suggests a link between poor outcome and specific types of *K-ras* mutations (codon 12 G-T transversions) in colorectal cancer (64). Demonstration of such a prognostic effect could allow appropriate targeting for intensive follow-up and adjuvant therapy (65,66). Detection of *K-ras* mutations may refine the assessment of liver metastases colorectal cancer (67). Although limited by a low sensitivity, detection of *K-ras* mutations in sputum, bronchoalveolar lavage, or pleural fluid may improve cytologic diagnosis in both early stage (68) and recurrent lung carcinomas (69). In patients with NSCLC, the presence of *K-ras* mutations has been related to an unfavorable prognosis (70).

## Raf

The *Raf* genes encode for Ser-Thr kinases involved in the transduction of mitogenic signals from the plasma membrane to the nucleus. The functions of the isoforms of Raf are partially overlapping and the absence of one member determines an increase of the activity of the two other isoforms (71). The three isoforms of Raf protein kinases identified are Raf-1 (also named c-Raf), A-Raf, and B-Raf. Overall, B-Raf shows >50% homology to both A-Raf and Raf-1 (72). The activity of each isoform is regulated in a specific way.

*B-Raf* is located on chromosome 6, and a 2.2-kb cDNA has been isolated (72). The presence of at least 10 transcripts, ranging from 2.2 to 12 kb due to alternate splicing, have been documented. All proteins arising from B-Raf mRNA alternatively spliced maintain intact kinase activity (73). The B-Raf sequence contains the three conserved regions: CR1, which encodes for the putative zinc-finger domain; CR2, where several Ser/Thr-rich regions are located; and CR3, which corresponds to the kinase domain. B-Raf is recruited to the plasma membrane on binding to Ras-GTP and represents a key point in the signal transduction through the MAPK pathway (Fig. 2).

B-Raf has an increased basal kinase activity due to Ser445 constitutively phosphorylated and to the presence of two Asp residues at positions 447–448. B-Raf is activated by Ras, through phosphorylation of Thr598 and Ser601 (74), by Rap-1 family genes, through lipid modifications of Rap1B, and by several growth factors (73). By contrast, B-Raf is negatively regulated through phosphorylation of Ser364, Ser428, and Thr439 (75), and by serum and glucocorticoid-inducible kinase on Ser364 (76).

Furthermore, it has been shown that cyclic adenosine monophosphate may activate or antagonize ERK through B-Raf (73). Binding of 14-3-3 protein to Ser621 of Raf-1 pro-

tein determines B-Raf/Raf-1 complex formation, a finding that may explain the cooperation between B-Raf and Raf-1 in cells on stimulation of several growth factors (77).

B-Raf represents the major MEK activator even in cells where its expression is detectable at very low level by Western blot analyses (reviewed in ref. 73). B-Raf is predominantly expressed in adult epididymis, ovary, testes, spinal cord, and cerebrum, and fetal membranes and brain (72). Proof of involvement of B-Raf in tumor development is a recent issue. It has been shown that B-Raf is responsible for proliferation and differentiation of cells in leukemia (78) and in melanoma cell lines (79). Screening of different cell lines and tissue samples provided the first detection of *B-Raf* mutations in tumors (80). In cell lines, *B-Raf* alterations were detected in melanoma (59%), colorectal cancer (18%), liver cancer (14%), and gliomas (11%). In tissue specimens, *B-Raf* mutations were found in melanomas (75%), colorectal cancer (12%), and ovarian cancer (14%). All mutations are located in exons 11 and 15, primarily due to the amino acid change Val to Glu in position 599. It is likely that the insertion of a negative charge in the proximity of residues 598 and 601 may determine Ras-independent constitutively activated B-Raf. *B-Raf* mutations occur in colorectal cancer only when tumors do not carry mutations in *K-Ras* gene, and such a mutual exclusion has lead to assume that *B-Raf* and *K-Ras* alterations could have the same functional effect in colorectal carcinogenesis (81). Moreover, again in colorectal cancer, it seems that *B-Raf* mutations could be related to MMR status (81).

## HER-2/neu

The *HER-2/neu* gene, part of the tyrosine kinase oncogene family, is located on chromosome 17q21 and encodes for a transmembrane receptor-like phosphoglycoprotein (185 kDa) that is closely related in structure but is biologically distinct from the epidermal growth factor receptor (EGFR) (82).

EGF and HER-2/neu receptors have a glycosylated extracellular N-terminus where the ligand binds, a hydrophobic transmembrane region, and a kinase domain contained within the intracytoplasmic C-terminus. The cytoplasmic domains of these receptors contain several tyrosines that can become phosphorylated on activation and bind to proteins that contain src homology 2 (SH2) domains and are part of the signal transduction pathway. A truncated form of HER-2/neu protein, lacking the N-terminal extracellular domain, was found to be associated with nodal metastasis in breast cancer (83). It is not known if this alteration is due to posttranslational cleavage or to alternative splicing mechanisms. Gene amplification and overexpression of Her-2/neu receptor have been reported in approx 25% of breast, ovarian, endometrial, gastric, and salivary gland carcinomas (84). This receptor was detected in 16% of NSCLC (85) and in a subset of malignant pancreatic endocrine tumors (86). Overexpression of the Her-2 receptor, at immunohistochemical level, was detected in thick melanoma (87), pancreatic carcinoma (88), and prostatic tumors (89). Methods for the detection HER-2/neu amplification include fluorescence *in situ* hybridization (FISH), Southern blot analysis, slot-blot hybridization, and quantitative polymerase chain reaction (PCR).

*HER-2/neu* has potential prognostic clinical applications in breast cancer: detection of *HER-2/neu* amplification has utility in identifying highly aggressive node-positive breast carcinomas (31,84,90). In patients with axillary node-negative breast cancer, *HER-2/neu* amplification has been reported as an independent prognostic factor for risk of recurrence (91). In endometrial cancer, *HER-2/neu* amplification has a potential



prognostic marker of poor outcome and may have clinical utility in selecting patients for adjuvant therapy (92). In NSCLC, a negative impact of *HER-2/neu* overexpression on survival was reported for patients who had radical resection (93). In brain tumors, *HER-2/neu* overexpression and short-term mortality were related in primary malignant brain tumors (94).

*HER-2/neu* gene expression has been reported as a new factor for the prediction of treatment responsiveness in breast cancer. In particular, information regarding expression of this oncogene may become important for the prediction of response to anthracycline-containing chemotherapy and resistance to treatment with tamoxifen or CMF (chemotherapy with cyclophosphamide, methotrexate, and 5'-fluorouracil [5-FU]) (95). In a study of 386 patients with breast cancer with a 20-yr follow-up, no differences in treatment response were found in patients with *HER-2*-positive or *HER-2*-negative tumors, suggesting that even patients with *HER-2*-negative tumors may benefit from chemotherapy treatment (96).

*HER-2/neu* has become a target for anticancer therapies. Trastuzumab, a monoclonal antibody (MAb) that binds the receptor, has been developed and used in clinical trials, prolonging survival of patients with metastatic disease overexpressing *HER-2/neu* (97). Trastuzumab has been used as adjuvant therapy in patients with *HER-2*-positive breast cancer (98). In NSCLC, ongoing phase 2 trials suggest that trastuzumab can be added to standard chemotherapy in these patients with promising efficacy (99).

## RET

The *RET* protooncogene is located on chromosome 10q11.2 and comprises 21 exons, which encode a receptor-type tyrosine kinase (RTK) that is involved in the control of neural crest cell proliferation, migration, survival, and/or differentiation. The Ret receptor comprises an extracellular ligand-binding domain (LBD) that contains a region of cadherin homology of unknown significance, a transmembrane domain, an intracellular tyrosine kinase domain, and additional amino acid sequences that function as regulatory domains (100). This receptor is expressed in three isoforms owing to alternative splicings involving the last three exons (101). Ligand binding induces receptor dimerization and autophosphorylation in a *trans* fashion and functions to recruit intracellular signaling proteins. *RET* expression occurs predominantly in neural crest-derived cells (100).

The *RET* protooncogene can be activated by two mechanisms: gene mutation (including missense and in-frame duplication) and rearrangement with genes that generate fusion proteins. Germline mutations in the *RET* protooncogene are associated with multiple endocrine neoplasia type 2 (2A and 2B) and familial medullary thyroid carcinoma. Somatic point mutations of *RET* have been described in 23–69% of sporadic medullary thyroid carcinomas (102). The most common somatic mutation occurs in the intracellular tyrosine kinase domain of *RET* at codon 918 within exon 16 and causes the substitution of a Met by a Thr.

Methods for detecting *RET* gene mutation include mutation-specific restriction enzyme analysis, double-gradient DGGE, direct sequencing, and oligonucleotide microarray. Potential clinical applications include diagnosis (analysis of *RET* mutations in both peripheral blood leukocytes and tumor specimens enables discrimination between hereditary and sporadic medullary thyroid carcinoma) and prognosis (tumor-specific mutation at codon 918 has been reported to correlate with tumor recurrence and poor prognosis) (103).

## BCL2

The *BCL2* (B-cell leukemia/lymphoma 2) gene, located on chromosome 18q21, spans more than 230 kb of DNA and consists of three exons of which exon 2 and a small part of exon 3 encode for protein. Dependent on splicing of intron 2, *BCL2* encodes for two mRNAs, *BCL2* $\alpha$  and *BCL2* $\beta$ , of which only *BCL2* $\alpha$  seems to have biologic relevance. The *BCL2* $\alpha$  protein is a 26-kDa membrane protein located at the cytosolic site of the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membrane. *BCL2* inhibits apoptosis under stress conditions and prolongs cell survival. In normal tissues, *BCL2* protein displays a restricted topographic distribution within mature tissues that are characterized by apoptotic cell death. In secondary follicles, *BCL2* is strongly expressed in mantle zone, which comprises long-lived recirculating cells. In the thymus, *BCL2* is present in the surviving mature thymocytes of the medulla. *BCL2* is usually expressed in hematopoietic precursor cells, but it is absent in their most differentiated and terminal progeny (104). *BCL2* is present in complex differentiating epithelium, where it is restricted to stem cell and proliferating zones. Because of its antiapoptotic function, the *BCL2* gene initiated a new category of oncogenes, called regulators of cell death. Recently, *BCL2* homolog, some of which bind to *BCL2*, have been identified, suggesting that *BCL2* functions at least in part through protein–protein interaction. Site-directed mutagenesis of *BCL2* protein BH1 and BH2 domains showed that these two regions are important for binding of *BCL2* to bax, a member of the *BCL2* family that promotes cell death and whose interaction with *BCL2* is necessary to regulate the apoptotic pathway.

The *BCL2* gene was discovered because of its involvement in the t(14;18) of follicular lymphomas. Although translocation is the main mechanism of *BCL2* gene activation, *BCL2* point mutations and amplification have been reported. Mutations clustering in the *BCL2* open reading frame occur in high-grade B-cell lymphomas transformed from low-grade follicular lymphomas carrying *BCL2* gene rearrangement (105). *BCL2* gene amplification, which leads to increased protein production, has been detected in approx 30% of high-grade diffuse large cell lymphomas (DLCL) lacking *BCL2* translocation (106).

*BCL2* expression has been investigated in both lymphoid and nonlymphoid tumors. In solid tumors, *BCL2* expression occurs in tumors of some hormonally responsive epithelia, such as breast, prostate, and thyroid. Detection of *BCL2* protein has diagnostic utility and some prognostic value. Detection methods include Southern blot hybridization (for gene amplification) and PCR-SSCP and direct sequencing for gene mutation; *BCL2* protein expression can be determined by immunohistochemistry (IHC), Western blot, or flow cytometry.

The clinical applications of *BCL2* include diagnosis of lymphomas in which *BCL2* protein expression is used as a marker for the differential diagnosis between reactive follicular hyperplasia and follicular lymphoma (107,108). Prognosis of patients with leukemia and lymphomas can be helped by *BCL2*. Because of the occurrence of *BCL2* gene mutations in transformed high-grade B-cell lymphomas (105), this genetic lesion may represent a predictive marker of progression in *BCL2*-rearranged tumors. Clinical correlation studies in DLCL indicated that *BCL2* amplification is associated with advanced-stage disease at presentation (109).

In high-grade B-cell lymphomas, *BCL2* protein expression is a strong, major predictor of overall survival, disease-free survival, and relapse-free survival either by itself (110) or in association with p53 expression (111), being related to a poor outcome.

High *BCL2* expression is associated with low remission rate in acute myeloid leukemia (AML) (112) and is an indicator of poor response in acute lymphocytic leukemia (ALL) (113). In neuroblastoma and carcinoma of the prostate, *BCL2* positivity is a poor-prognostic marker, whereas breast cancer patients with *BCL2*-positive tumors have improved survival (114). In the thyroid, *BCL2* is overexpressed in adenomas and well-differentiated carcinomas and is frequently completely lost in anaplastic carcinoma (115,116). In medullary thyroid carcinoma, lack of *BCL2* immunoreactivity correlated significantly with a shorter survival (117); therefore, down regulation of *BCL2* expression in this disease may identify a subset of tumors with a more aggressive clinical course. The association between IHC staining for *BCL2* protein and the histologic type and prognosis of NSCLC is controversial (118). *BCL2* expression may be involved in the metastatic progression of pancreatic carcinoma (119).

In cancers that overexpress *BCL2*, decreasing its expression, by targeting *BCL2* directly or indirectly through an upstream regulator of *BCL2*, may render the neoplastic cells more sensitive to chemotherapeutic agents. The downregulation of *BCL2* protein expression by antisense oligonucleotides that induce *BCL2* mRNA degradation may offer a new therapeutic approach for treatment improvements in patients with neoplasms resistant to traditional therapies. The results with in vitro (120,121) and in vivo (122) models are promising, especially when combination of *BCL2* antisense oligonucleotides (G3139) with a cytotoxic agent is used. In humans, G3139 has been studied as a single agent in a phase 1 trial in 21 heavily pretreated patients with relapsed non-Hodgkin's lymphoma (NHL) (123), in which one complete and two minor responses and nine disease stabilizations were observed. One study combining a chemotherapeutic agent with G3139 has been reported in metastatic melanoma (124), and studies are ongoing in a number of other solid tumors (melanoma, prostate carcinoma) and hematologic malignancies (myeloma, chronic lymphocytic leukemia [CLL], and AML).

### **BCL1-PRAD1-CCND1**

The *PRAD1* gene, which was first cloned from a parathyroid adenoma with inv(11) (p15;q13) and subsequently identified as *BCL1*, maps on chromosome 11q13. Transcription produces two mRNAs of 4.5 and 1.5 kb through alternative polyadenylation. The *BCL1* gene encodes for a 36-kDa nuclear protein of 295 amino acids, cyclin D1, which belongs to the cyclin G1 family. The *BCL1*-cyclin D1 protein binds and activates the CDK4 and CDK6 and seems to regulate the cell cycle G1-S-phase checkpoint through phosphorylation of Rb (125). In normal tissue, the *BCL1*-cyclin D1 protein is expressed in the proliferating fraction of epithelial tissues, and is absent in lymphoid tissues such as lymph node, spleen, and tonsil (126).

The main mechanisms of *BCL1* gene activation include translocation and amplification, both of which result in overexpression of normal RNA of 1.5 and 4.5 kb and of intact 36-kDa cyclin D1 protein. The 11q13 region is involved in B-cell lymphomas, parathyroid adenoma, breast cancer, and squamous cell cancer of the head and neck. Detection methods include Southern blot analysis for amplification, and Northern blot, reverse transcriptase PCR (RT-PCR), real-time quantitative PCR, RNA *in situ* hybridization, Western blot, and IHC for overexpression.

The *BCL1* gene has diagnostic applications in patients with lymphoma. *BCL1* protein expression is a marker that enables a differential diagnosis of mantle cell lymphoma, being positive in >80% of cases (127).

In solid tumors, prognosis can be determined by the *BCL1* gene. In cancers of the breast and of the head-and-neck region, 11q13 amplification is associated with poor clinical course of the disease (128): the relapse-free survival time of patients with *BCL1*-amplified breast tumors was shorter than that of patients without *BCL1* alteration. In a study in patients with esophageal carcinoma (129), *BCL1* was amplified in a subset of primary tumors and lymph node metastases. Metastases were more common in patients with *BCL1* amplification than in those without this abnormality. Moreover, *BCL1* amplification was associated with decreased 1-yr survival, thus providing useful prognostic information.

## REL

The *REL* gene, the human homolog of the reticuloendotheliosis virus strain T (REV-T), which induces leukemia in chickens, was identified in a human genomic DNA library and assigned to chromosome 2. Using FISH, *REL* was mapped to the 2p15;14 position (130). *REL*, which consists of seven exons, belongs to the Rel/nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcriptional activators that can heterodimerize and participate in cytoplasmic/nuclear signal transduction in response to cytokines, mitogens, physical and oxidative stress, and other pathogenic products (131). In humans, high concentrations of *REL* mRNA are found in relatively mature lymphocytes. Its expression is depressed in immature thymocytes and may have a role in lymphocytic differentiation (132).

The Rel/NF- $\kappa$ B transcription factors serve an important regulatory role in inflammation, the immune response, and the cellular stress response and have been implicated in control of cell proliferation and oncogenesis. Blocking of Rel/NF- $\kappa$ B signaling rapidly develops spontaneous skin cancers (133). The *REL* oncogene has been found amplified in a subset of DLCL (131) and in a proportion of classical cases of Hodgkin's disease, in which, by FISH analysis, signal patterns suggestive of breakpoints in the region spanned by the *REL* probe were observed (134). Detection methods include Southern blot hybridization and FISH.

Potential clinical applications include diagnosis and prognosis. A 35-fold *REL* amplification has been described in approx 20% of patients with DLCL, >70% of which are primary extranodal lymphomas (109). The occurrence of this abnormality in advanced-stage disease, and the association with other genetic lesions, suggest that *REL* may represent a progression-associated marker of primary extranodal lymphomas.

## APC

*APC* (adenomatous polyposis coli) is a TSG encoding for a large multidomain protein that plays a relevant role in the Wnt-signaling pathway and intercellular adhesion. *APC* consists of 8535 bp spanning 21 exons on chromosome 5q21. Exon 15 includes >75% of the coding sequence. mRNA arising from alternate splicing has been described (135).

The *APC* protein comprises several functional domains, including:

- Heptad repeats at the N-terminal region that mediate *APC* homodimer formation.
- Domain binding to Asef to control cell adhesion and motility.
- Two motifs, an imperfect repeat of 15 amino acids and an imperfect repeat of 20 amino acids (containing glycogen synthase kinase 3 $\beta$  [GSK3 $\beta$ ] consensus site), which interact with  $\beta$ -catenin (codons 1020–2033) and interspaced with SAMP motifs.

The basic domain, which binds to microtubules, is located at the C-terminal region (136). *APC* alterations can be detected by IHC, protein truncation test, DNA promoter hypermethylation assay, sequencing, and allelic imbalance.

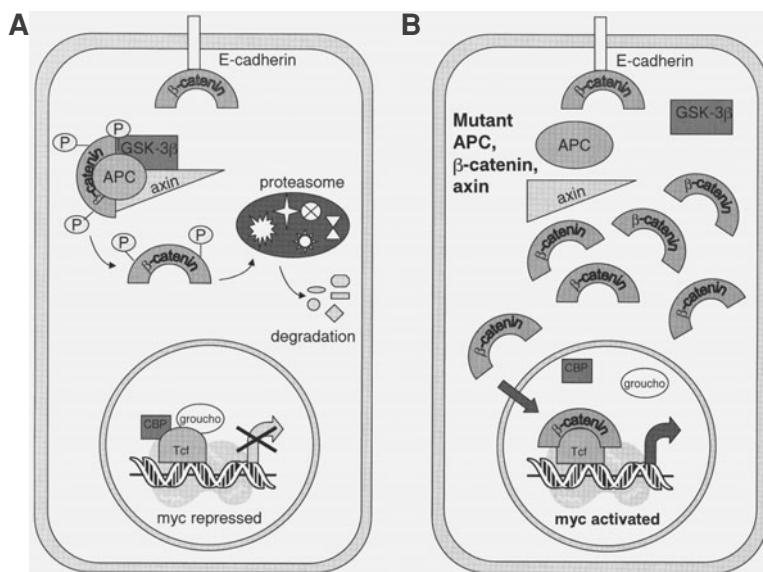


Fig. 3. APC/β catenin pathway and myc overexpression in normal (A) and tumoral (B) cells.

Somatic mutations result in both loss of the wild-type APC allele and mutations that cumulatively occur in >80% of colorectal cancers. The somatic mutations encompass nonsense, nucleotide insertions, and deletions (all of which cause a premature stop codon determining an abnormal truncated protein) and can occur anywhere. More than 60% of somatic mutations, however, occur between codons 1286 and 1559, a region named mutation cluster region (MCR). Within the MCR, two hot-spot mutations are at codon 1309 and 1450 (137). APC mutations in the MCR are associated with allelic loss while tumors with non-MCR mutations are coupled with truncating mutations, suggesting a strong selective pressure on the second hit of inactivation (138).

Germline mutations in APC have been seen in most patients with familial adenomatous polyposis (FAP). The most common germline mutations occur at codons 1061 and 1309. Severe polyposis is associated with mutations between codons 1250 and 1464, especially at the 1309 position. Attenuated polyposis frequently occurs when germline mutations are located at the N-terminal region of the APC gene. Extracolonic manifestations (i.e., desmoids, osteomas, upper gastrointestinal [GI] polyps) tend to occur with germline mutations located between codons 1445 and 1578 (136).

Although evidence for APC as an oncogene is very limited, its action as a homodimer suggests that dominant-negative mutations might occur (139). APC mutations could cause chromosomal instability (140).

APC plays a pivotal role in early phases of colorectal cancer by modulating β-catenin/Tcf transcriptional activation. In normal cells, APC is able to form a multiprotein complex with GSK3β and axin, is phosphorylated by GSK3β and binds to β-catenin, which in turn is phosphorylated by GSK3β and subsequently degraded by the proteasome pathway (Fig. 3). In tumoral cells, when APC (as well as β-catenin or axin) is mutated, the multiprotein complex could not be formed, and, therefore, β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it activates Tcf factor, which in turn causes transcription of target genes, such as *c-myc* (141). APC may have an indirect role in the regulation of apoptosis (136).

The relevance of *APC* during colorectal carcinogenesis renders such a gene a good candidate for early diagnosis in bodily fluid, such as plasma, blood, or stool. A sensitive method to detect *APC* alterations in stool specimens has been developed, but it is very difficult and time-consuming for routine laboratory work (142).

## MYC

MYC is a member of the helix-loop-helix/leucine zipper superfamily, a gene family containing at least seven closely related genes. The most studied are *C-MYC* (cellular), *N-MYC* (originally isolated from neuroblastoma cells), and *L-MYC* (originally isolated from small-cell lung cancer [SCLC] cells). The *MYC* genes encode for nuclear DNA-binding proteins that are involved in transcriptional regulation. MYC proteins form homodimers or heterodimers through their C-terminal helix-loop-helix domains. MYC can heterodimerize with proteins such as max, mad, and MX11. max can bind MYC to repress the transcriptional activation of *MYC* genes, whereas mad and MX11 can bind max and release MYC to function as a transcriptional activator (52). MYC is implicated in the control of normal cell proliferation, transformation, and differentiation. MYC expression is essential for progression through the cell cycle and is growth factor dependent in untransformed cells (143). A biologic function of MYC in different cell types is apoptosis induction in the absence of specific growth factors.

*C-MYC*, the major member of the *MYC* family, is located on human chromosome 8q24 and consists of three exons, the first of which is noncoding. *N-MYC* and *L-MYC* map on chromosome 2p24.1 and 1p32, respectively. *MYC* abnormalities can be in the form of chromosome translocation, gene mutation, or gene amplification. The result is usually increased *MYC* expression rather than change of the protein structure. Furthermore, *MYC* activation may be mediated by APC and/or  $\beta$ -catenin alterations in several tumors, leading to an increase in *MYC* transcription through an accumulation of  $\beta$ -catenin into the cytoplasm and the nucleus (Fig. 3) (144). Tumor-associated *MYC* alteration may be related to an imbalance of the myc/max system (145).

In lymphomas, *C-MYC* gene mutations can occur in the gene transactivation domain and in the coding region after translocation into the *Ig* gene (146). Mutations can occur in the noncoding gene exon 1 and at the exon 1/intron 1 boundary with or without *C-MYC* gene translocation (146). This region is considered the *C-MYC* regulatory region and is responsible for its mRNA stability. In Burkitt's lymphoma, mutations frequently occur at sites of phosphorylation, which suggests that they may have a role in pathogenesis.

*C-MYC* deregulation is associated with its amplification in DLCL, SCLC, and ovarian and breast carcinoma. *N-MYC* is frequently amplified in SCLC, neuroblastoma, and retinoblastoma. *L-MYC* deregulation is related to its amplification in lung cancer and to loss of heterozygosity (LOH) in bladder and lung cancer. Methods for detecting *MYC* alterations include Southern blot, FISH, and PCR for amplification, and PCR-SSCP and direct sequencing for *MYC* mutations.

In patients with DLCL, *C-MYC* amplification, in association with other genetic lesions, occurs in approx 20% of cases and is considered a progression marker (109). A nuclear accumulation of c-myc may identify high-risk subsets of patients with synovial sarcoma of the extremities (147).

*C-MYC* amplification and overexpression, in combination with *HER-2/neu* alterations, have been reported to be associated with tumor progression from noninvasive to invasive (148) and with poor prognosis (90) in patients with breast carcinoma. A recent

study provides evidence that C-MYC immunoreactivity may be a predictor of poor prognosis and poor response to radiotherapy in carcinoma of the cervix (149), a finding not otherwise confirmed by other reports (150). In melanoma (151) and in medulloblastoma (152,153), C-MYC expression seems to be a useful prognostic marker able to identify high-risk patients.

It has been proposed that *N-MYC* might regulate angiogenesis because its overexpression leads to a downregulation of leukemia inhibitory factor, a modulator of endothelial cell proliferation (154). In neuroblastoma, *N-MYC* amplification has been correlated with increased metastases and poor outcome (155). Contradictory findings exist about the association between *N-MYC* amplification and *BCL2* expression in neuroblastoma (155,156).

Human genomic DNA shows an *EcoRI* RFLP of *L-MYC* defined by two alleles, S and L. RFLP is a representative genetic trait associated with an individual's susceptibility to several tumors such as gliomas and esophageal and gastric cancer. Otherwise, the association between RFLP and lung cancer prognosis has produced conflicting results due to racial/ethnic and methodologic differences (157,158). A significant reduction in *L-MYC* expression may be associated with disease stage and course in bladder cancer (159).

## BCL6

The *BCL6* gene (also known as *BCL5* or *LAZ-3*) maps on chromosome 3q27 and consists of nine exons, the first two of which are noncoding. The gene is transcribed as a 3.8-kb message predominantly in normal adult skeletal muscle and in some patients with NHL carrying 3q27 chromosomal defects. *BCL6* encodes for a 79-kDa nuclear protein containing six C-terminal zinc-finger domains and an N-terminal POZ domain, which mediates its sequence-specific transcriptional repressor function (160). The *BCL6* protein is predominantly expressed in the B-cell lineage, where it is found in mature B cells. In normal human lymphoid tissues, *BCL6* expression is topographically restricted to germinal centers, including all centroblasts and centrocytes. This restriction indicates that *BCL6* is specifically regulated during B-cell differentiation and suggests a role for *BCL6* in germinal center development and function (161,162).

The *BCL6* gene can be activated by chromosomal translocation or somatic mutations. Breakpoints and mutations cluster in the *BCL6* regulatory region, in a 3.3-kb *EcoRI* fragment that defines the major translocation cluster. *BCL6* somatic mutations are multiple and often biallelic and are found in tumors displaying either normal or rearranged *BCL6* alleles, indicating their independence from chromosomal rearrangement and from linkage to *Ig* genes. *BCL6* gene mutations have been found in >70% of DLCLs and 45% of follicular lymphomas (163). Detection methods for *BCL6* somatic mutations include PCR-SSCP and direct sequencing.

*BCL6* mutations have been found in a high proportion of normal B-cells (164,165) and in most germinal center-derived lymphomas (166), so this genetic abnormality does not seem to have a diagnostic utility. Transformation of follicular lymphomas to DLCL is associated with accumulation of new mutations in the 5' noncoding regulatory region of *BCL6* that may regulate *BCL6* mRNA expression (167) and, in some cases, have a role in lymphoma transformation. From the prognostic point of view, it has been shown that *BCL6* mutations predict shorter survival and refractoriness to reduced immunosuppression and/or surgical excision in posttransplantation lymphoproliferative disorders. Investigation of the prognostic value of *BCL6* mutations is at early stages (168). In keeping with this, the presence of *BCL6* gene mutation could

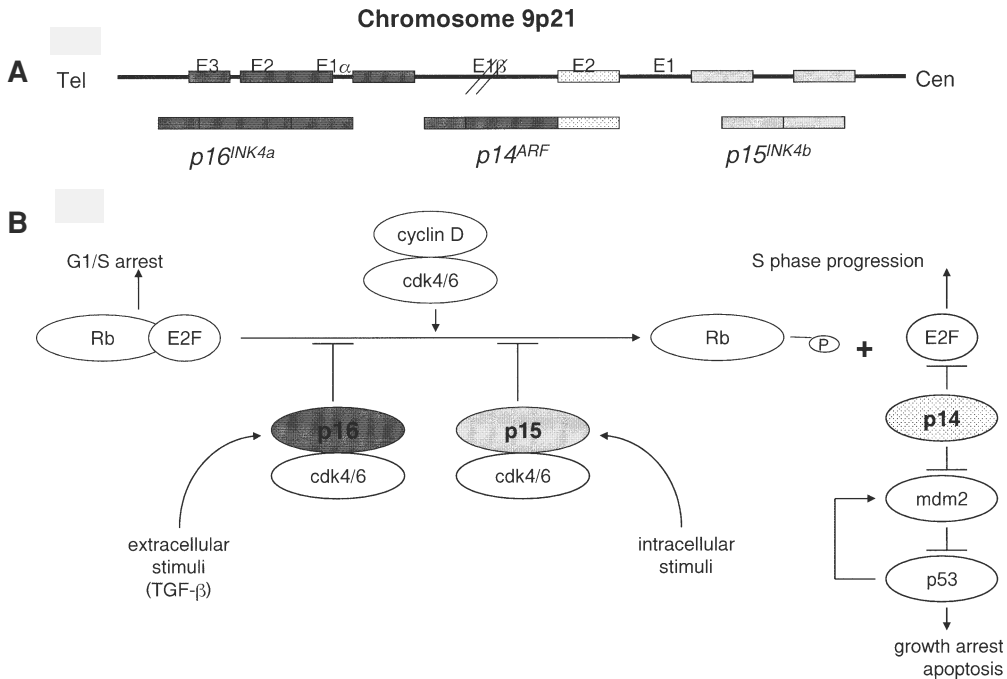


Fig. 4. (A) Structure and (B) tumor-suppressive pathways at 9p21 locus.

predict a higher chance of being free of disease in patients with DLCL treated with standard chemotherapy, but not in those treated with autologous stem cell transplantation (169). High BCL6 mRNA expression may be a favorable prognostic factor in DLCL (170), and its use in the stratification and design of risk-adjusted therapies for patients with DLCL is encouraged.

### 9p21 Chromosomal Region

The 9p21 chromosomal region harbors a gene cluster consisting of three physically proximate genes: *p14<sup>ARF</sup>*, *p16<sup>INK4a</sup>* and *p15<sup>INK4b</sup>*. *p14<sup>ARF</sup>* and *p16<sup>INK4a</sup>* both are encoded by the *INK4a/ARF* locus that shows a peculiar genomic organization containing two distinct promoters and alternative first exons, designated 1 $\alpha$  and 1 $\beta$ , whose transcripts are each spliced to two common exons. Exons 1 $\alpha$ , 2, and 3 encode *p16<sup>INK4</sup>*, while exons 1 $\beta$ , 2, and 3 encode *p14<sup>ARF</sup>* that bears no homology to *p16<sup>INK4</sup>*. *p15<sup>INK4b</sup>* gene maps in tandem in chromosome band 9p21, 25 Kbp upstream of the *p16* gene (Fig. 4A).

All three genes have a putative tumor suppressor role; *p14<sup>ARF</sup>* is a key component of the *TP53* pathway, whereas *p16<sup>INK4a</sup>* and *p15<sup>INK4b</sup>* play an active role in the *Rb* pathway. On the basis of their cell-cycle inhibitor function, alterations of each gene can influence cellular growth regulation (Fig. 4B).

9p21 chromosomal band is one of the major aberration hot spots in human cancers (171), and its high susceptibility to genetic alterations is probably related to physical organization of this gene cluster. The existence of tightly clustered breakpoints (close to the 1 $\alpha$  and 1 $\beta$  exons and possibly also upstream of *p15<sup>INK4b</sup>*) in the 9p21 locus, as well as gene-specific deletion by illegitimate V(D)J recombinase activity (172), has



been reported. Furthermore, the promoter regions of the three genes are rich in CpG islands that are targets for gene silencing by methylation.

### p16<sup>INK4a</sup>

p16<sup>INK4a</sup> (also known as MTS1 for major tumor suppressor 1, INK4a for inhibitor of CDK-4a, and CDKN2A for CDK-inhibitor 2A) is a G1-specific negative regulator of cell proliferation. The *p16<sup>INK4a</sup>* gene comprises three exons coding for a 15.8-kDa protein of 156 amino acids that have a four tandemly repeated motif structure. The p16 protein is the prototype of a family of nonfunctional-redundant CDK inhibitors (i.e., *p15<sup>INK4b</sup>*, *p18<sup>INK4c</sup>*, *p19<sup>INK4d</sup>*). Their function is to block the association of CDK4/6 with cyclin D and prevent the activation of the kinase activity of the CDK4/6–cyclin D complex. The CDK4/6–cyclin D complex can phosphorylate the pRB protein that concomitantly releases E2F, a factor that permits transcription of the cell-cycle regulator genes and then progression into S-phase. Binding of CDK4 or 6 with p16 protein blocks cell cycle in G1. These functional relations are known as the p16<sup>INK4a</sup>/CDK4/cycD1/Rb pathway.

The amount of p16 mRNA in normal human tissue is quite low, but accumulation of p16 transcript and protein has been shown in response to cellular senescence, oncogenic *RAS* gene stimulus, and inactivation of *Rb* gene.

To date, three mechanisms of genetic inactivation of *p16<sup>INK4a</sup>* gene have been found: deletion of both alleles, deletion of one allele and mutation in the remaining allele, and deletion of one allele and methylation-mediated silencing of the remaining allele (173).

Allele inactivation is detected by Southern blot, real-time PCR, and quantitative and microsatellites analysis. To determine LOH, normal tissue comparison is required. Intragenic mutations can be uncovered by mutation analysis (SSCP and direct sequencing); promoter methylation is performed by methylation-specific-PCR that requires bisulfite modification of DNA and primers specific to methylated and unmethylated template.

Deletions, point mutations, and methylation of the 5' CpG island are molecular abnormalities that could affect p16<sup>INK4a</sup> function in many human cancers. Specifically, *p16<sup>INK4a</sup>* point mutations commonly occur in pancreas, esophageal, lung, and head-and-neck carcinomas. Deletions of *p16<sup>INK4a</sup>* have been identified in melanoma, bladder and prostate carcinoma, T-cell ALL, glioma, mesothelioma, sarcoma, and ovarian and renal cell carcinoma. Methylation of the 5' CpG island was found to be associated with breast, colon, bladder, head-and-neck, lung, and brain tumors.

Potential clinical applications include risk assessment. LOH as well as mutations of *p16<sup>INK4a</sup>* have been reported in sporadic dysplastic nevi, suggesting their role in the development of malignant melanoma (174).

In diagnosis, sporadic pancreatic cancers have been found affected by *p16<sup>INK4a</sup>* mutations, which seem to be useful as diagnostic markers, but their biologic meaning has not been defined (175). 9p21 LOH and *p16<sup>INK4a</sup>* alterations were significantly related to shorter survival, quicker relapse, and worse prognosis in laryngeal squamous cell carcinoma (176), ductal pancreatic cancer (177), and Ewing's sarcoma (178). Evidence of a role for *p16<sup>INK4a</sup>* inactivation in tumor progression was reported in meningioma (179).

In hematopoietic tumors, *p16<sup>INK4a</sup>* inactivation has frequently been observed (e.g., ALL and some B- and T-cell lymphomas, and NHL). Adults with B- or T-cell lymphoma who carry such an alteration probably have a poor prognosis (180). Paired sequential analyses showed that transformation from low- to high-grade B-cell lym-

phoma is associated with loss of  $p16^{INK4a}$  activity (181), suggesting that  $p16^{INK4a}$  may be involved in progression and may represent a target for new therapeutic strategies. Alterations of p16 protein expression are not relevant in term of diagnostic or prognostic significance. Nevertheless, lack of p16 expression and p16 overexpression may be of prognostic importance in NSCLC (182) and in neuroblastoma (183), respectively.

$p16^{INK4a}$  inactivation correlates with poor treatment outcome both in patients and in mouse models. The action of traditional drugs, inducing DNA damage and apoptosis, depends on effective programs of senescence and apoptosis controlled by p16 and p53 (184). Thus, the molecular characterization of  $p16^{INK4a}$  could be helpful in planning a more tailored cancer therapy.

### p15INK4b

$p15^{INK4b}$  gene displays high homology to  $p16^{INK4a}$ , particularly in exon 2, indicating its origin by a gene duplication event. The gene comprises two exons and generates two distinct mRNAs, p15 and p10. p15 transcript encodes two protein isoforms, p15 and p15.5, that are synthesized from two alternative, in-frame, translation initiation codons. Although structurally distinct, both the proteins bind to CDK4 and CDK6 and suppress cell growth in response to extracellular stimuli, such as transforming growth factor (TGF- $\beta$ 1), at variance with p16 that is activated by intracellular stimuli. The role of  $p15^{INK4b}$  in human tumorigenesis and definitive conclusions regarding the prognostic significance of its inactivation remain to be better defined. This gene is inactivated by homozygous deletion, possibly involving contiguous  $p16^{INK4a}$  and  $p14^{ARF}$ , in tumors such as esophageal squamous cell carcinoma (185), ALL (186), and bladder carcinoma (187). In ovarian cancer,  $p15^{INK4b}$  deletion may be a potential indicator of poor chemotherapy response and adverse prognosis (188). Similarly, in adult T-cell leukemia/lymphoma, patients with deleted  $p15^{INK4b}$  and  $p16^{INK4a}$  have a significant shorter survival than those individuals with both genes preserved (189). A highly significant association between homozygous deletion of  $p15^{INK4b}/p16^{INK4a}$  and the two major adverse prognostic factors (i.e., T-cell immunophenotype and first remission duration) has been found in relapsed childhood ALL (190).

Mutations of  $p15^{INK4b}$  are not frequent, whereas promoter methylation was reported in some cancers as neuroendocrine lung tumors (191), ALL, and AML (192). In acute promyelocytic leukemia (APL) (193) and in precursor B-cell ALL (PBC-ALL) (194),  $p15^{INK4b}$  (but no  $p16^{INK4a}$ ) gene methylation is a potential marker of minimal residual disease and may be of prognostic significance. The possible correlation between  $p15^{INK4b}$  inactivation by methylation with an aggressive transformation of B-cell and T-cell lymphomas (195), or an evolution of myelodysplastic syndromes toward AML (196), provides evidence of a causal role of the promoter methylation in disease progression.

### p14<sup>ARF</sup>

In a  $p14^{ARF}$  (also known as ARF [alternative reading frame]) null mice model, lymphomas and sarcomas develop at an early age, supporting the idea that human  $p14^{ARF}$  functions as TSG. At variance with  $p16^{INK4a}$  and  $p15^{INK4b}$ , in mouse and human cells,  $p14^{ARF}$  does not directly inhibit CDK but stabilizes p53 by antagonizing its negative regulator, mdm2. It has been accepted that  $p14^{ARF}$  binding to mdm2 induces a conformational change that facilitates nucleolar import of the  $p14^{ARF}$ -mdm2 complex. This interaction prevents mdm2-mediated p53 degradation by nuclear ubiquitination lead-

ing to a p53-dependent cell-cycle arrest or apoptosis depending on the cellular context. Contrasting results have been reported about the interplay between p14 and p53 in human cells. A p53-dependent cell-cycle arrest induced by p14 is sustained by some researchers (197), while others support that the p14 overexpression may be enough to induce a p53-independent apoptosis (198). Moreover, the occurrence of p14<sup>ARF</sup> multiple-binding domains for E2F, which negatively affect the E2F-dependent transcription, speaks in favor of a role of p14<sup>ARF</sup>, even in the Rb pathway (199).

The role of p14<sup>ARF</sup> in human carcinogenesis is less clear than that of p16<sup>INK4a</sup>, because alterations of p14<sup>ARF</sup> are accompanied by p16<sup>INK4a</sup> deregulation in most cancers. Alterations exclusively of p14<sup>ARF</sup>, as point mutations uniquely targeting exon 1 $\beta$ , are rare. Nevertheless, >40% of the *INK4a/ARF* mutations functionally impaired both p14<sup>ARF</sup> and p16<sup>INK4a</sup>, altering the subcellular distribution of p14<sup>ARF</sup> and decreasing its ability to activate the *TP53* pathway in melanoma (200).

Homozygous deletion is the predominant mechanism of p14<sup>ARF</sup> inactivation in hepatocellular carcinoma (201) and primary central nervous system (CNS) lymphomas where this specific alteration is associated with a shorter patient survival (202). Moreover, an increase in the homozygous deletion of p14<sup>ARF</sup>, always associated with codeletion of p16<sup>INK4a</sup>, correlates with a grade increase in primary gliomas (203).

A significant p14<sup>ARF</sup> promoter methylation was reported in oligodendroglial tumors (204), as well as breast (205) and colorectal cancers (206), and appears to be a biomarker for early detection of ulcerative colitis-associated colorectal cancer or dysplasia (207).

Regarding protein expression, in aggressive B-cell lymphomas an abnormal p14 nuclear overexpression, not confined to the nucleoli and associated with *TP53* and p16<sup>INK4a</sup> alterations, is a marker of a highly aggressive tumor and parallels an increased growth fraction as well as a more aggressive clinical course (208).

A peculiar p14<sup>ARF</sup> inactivation mechanism is represented by the t(8;21) chromosomal translocation, whose fusion protein, consisting of the AML-1 transcription factor and the 8-21 corepressor (AML1 ETO), represses the p14<sup>ARF</sup> promoter transcription and reduces endogenous levels of p14 expression in AML (209).

## c-kit

The *c-kit* protooncogene represents the cellular homolog of *v-kit*, an oncogene derived from the acute transforming feline retrovirus HZ4-FeSV (Hardy Zuckerman 4 feline sarcoma virus) (210). *c-kit* was mapped by *in situ* hybridization on chromosome 4, to locus 4cen-q21 (211). The gene spans >70 kb of DNA and includes 21 exons (212). The longest transcript is 5230 bp and is alternatively spliced.

The protooncogene *c-kit* encodes a 145-kDa transmembrane tyrosine kinase glycoprotein. As a member of the RTK subclass III family, *c-kit* is closely related to the receptors for the platelet-derived growth factor (PDGF), macrophage colony-stimulating factor (M-CSF), and FLT3 ligand. The juxtamembrane and kinase domains of these receptors are strongly conserved. *c-kit* is expressed by hematopoietic progenitor cells, mast cells, and germ cells, and by the pacemaker cells of the gut. Steel factor is also known as *c-KIT* ligand or stem cell factor (SCF). Binding of SCF to *c-KIT* results in receptor homodimerization, activation of *c-KIT* tyrosine kinase activity, and resultant phosphorylation of a variety of substrates, including AKT and STAT3. In many cases, these substrates are themselves kinases and serve as effectors of intracellular signal transduction.

*c-kit* mRNA expression has been found in a number of human malignancies, including mastocytosis/mast cell leukemia, germ cell tumors, SCLC, GI stromal tumors (GISTs), AML, neuroblastoma, melanoma, head-and-neck carcinomas, ovarian carcinoma, and breast carcinoma (213). Three general mechanisms of *c-kit* activation in tumor cells have been described: autocrine and/or paracrine stimulation of the receptor by its ligand, SCF; cross-activation by other kinases and/or loss of regulatory phosphatase activity; and acquisition of activating mutations.

The tyrosine kinase activity of *c-kit* can be activated by mutation of several exons of the gene. These activating mutations cause ligand-independent kinase activity with resultant receptor autophosphorylation and stimulation of downstream signaling pathways, including MAPK and phosphatidyl inositol 3' kinase (PI3K). *c-kit* mutations are most commonly found in mastocytosis/mast cell leukemia, AML, seminoma/dysgerminoma, and sinusoidal natural killer/T-cell lymphoma. In all these tumors, mutations involve codon 816 in exon 17. In GIST, more heterogeneous mutations are described, occurring in exons 11, 9, 13, and 17, comprising point mutations, "in-frame" insertions, and deletions. Detection methods include for *c-kit* mutations PCR or DHPLC and direct sequencing; for the autocrine or paracrine loop, methods include selective PCR for both ligand and receptor.

STI571, an inhibitor of tyrosine kinase activity in *BCR/ABL*-positive leukemia, is effective in treating GIST (214). In this pathology, gain of function mutations result in the ligand-independent receptor activation. STI571 (imatinib) was approved by the Food and Drug Administration in February 2002 for the treatment of GIST (215). A clinical phase 2 study demonstrated that patients with a GIST harboring an exon 11 mutation had a significantly higher partial response rate (72%) than patients whose tumor had an exon 9 mutation (31.6%) or no detectable mutation (11.8%).

## FLT3

*FLT3* gene, also known as *FLK-2* and *STK-1*, maps on chromosome 13q12 and belongs to the RTKIII receptor family. Like other members of this family, such as PDGF receptor (*PDGFR*) and *c-kit*, *FLT3* is characterized by an extracellular domain comprising five Ig-like domains and by a cytoplasmic domain with a split tyrosine kinase motif. In the human, *FLT3* encodes a 993 amino acid protein expressed in immature hematopoietic cells, placenta, gonads, and brain. By immunoprecipitation studies, two bands can be identified: a major band of approx 140 kDa and a less abundant band of approx 160 kDa, localized to the plasma membrane and derived by post-translational N-linked glycosylation of the smaller one (216). In normal bone marrow, *FLT3* expression appears to be restricted to early progenitors, including CD34<sup>+</sup> cells with high levels of CD117 expression. *FLT3* is highly expressed in a spectrum of hematologic malignancies, including 70–100% of AML, B-cell precursor ALL, a fraction of T-ALL, and chronic myelogenous leukemia (CML) in lymphoid blast crisis (217). A common mechanism of *FLT3* activation consists of internal tandem duplication in the juxtamembrane domain, which contains the inhibitory signal for the tyrosine kinase. Internal tandem duplication leads to length polymorphism in the juxtamembrane region. Alternative and less frequent mutations are localized in the activation loop of *FLT3*, which normally blocks access of adenosine triphosphate (ATP) and substrate to the kinase domain. Together, these mutations are present in approx 30% of AML patients and result in constitutive activation of the *FLT3* kinase (218). *FLT3* length mutations occur more frequently in AML patients with normal

karyotype (70%); among patients with chromosomal aberrations, they are found in those carrying t(15;17) (35%), and rarely in patients with t(8;21), inv(16), and complex karyotypes. In a study of a large cohort of AML cases, the complete remission rate and overall survival were not different between patients with or without FLT3-LM (219). By contrast, patients with FLT3-length mutations had a significantly shorter event-free survival with a higher relapse rate. FLT3-length mutations can represent a new genetic marker in AML to monitor a subset of patients during follow-up, predict relapse, and plan early therapeutic intervention. Alternative therapies for FLT3-length mutation could be considered; although not suitable for clinical trials in humans, selective *FLT3* inhibitors, such as AG1296, or herbimycin A gave interesting results in in vitro and in vivo models, respectively. Moreover, CEP-701 and CT53518 *FLT3* inhibitors are likely to move into phase 1 clinical trials because of their inhibiting properties on FLT3-internal tandem duplication (218).

### ***PTEN***

*PTEN* (phosphatase protein homolog to tensin deleted on chromosome 10), previously named *MMAC1* (mutated in multiple advanced cancers) or *TEP1* (TGF- $\beta$ -regulated and epithelial cell-enriched phosphatase), locates on the 10q23.3 region (220). *PTEN* is a TSG that encodes for a 403 amino acid protein that possesses lipid and protein phosphatase activities. Somatic mutations or loss of region 10q23 are involved in the development of several human tumors (221). With the important exception of endometrial cancer, in which *PTEN* plays a relevant role in early phases of carcinogenesis (222), *PTEN* seems to be involved in late stages of several tumors (220). Certain inherited disorders, such as hamartomatous syndromes, are associated with mutations of *PTEN* gene. These syndromes include Cowden disease, Lhermitte-Duclos disease, and Bannayan-Riley-Ruvalcaba disorder. The involvement of *PTEN* mutations in juvenile polyposis coli syndrome is conflicting (220). Evaluation of *PTEN* status is made by IHC, Northern blot, semiquantitative RT-PCR assay, LOH, homologous deletion, mutational analysis and methylation assay.

The transcriptional analysis of *PTEN* is difficult for the presence of a pseudogene of *PTEN*, named  $\Psi$ *PTEN* located on the 9p21 region. *PTEN* and  $\Psi$ *PTEN* mRNAs are both transcribed in all human tissues, and  $\Psi$ *PTEN* mRNA represents about 50% of *PTEN* mRNA. A method has been developed to analyze transcriptional *PTEN* expression without misinterpretation due to the presence of  $\Psi$ *PTEN* mRNA (223). To date,  $\Psi$ *PTEN* protein has not been detected, leading to the conclusion that analyses of *PTEN* protein expression do not show traces of contamination of  $\Psi$ *PTEN* protein. *PTEN* mutations are associated with the development of breast carcinomas in patients with Cowden's disease and lipoma in the Bannayan-Riley-Ruvalcaba syndrome (220). *PTEN* deletions and mutations are both frequent in sporadic endometrial carcinoma. By contrast, deletion of the *PTEN* region is a predominant event in melanoma and glioblastoma (224), and *PTEN* inactivation is a rare event in sporadic colorectal cancer (225). In addition to mutations and allele losses, other molecular mechanisms could determine *PTEN* inactivation. *PTEN* promoter hypermethylation occurs in approx 20% of endometrial carcinomas (226).

*PTEN* possesses both lipid and protein phosphatase activities. The phosphatase domain is composed of a central five-stranded  $\beta$ -sheet that packs with two  $\alpha$ -helices (227). *PTEN* can bind to cellular membranes, and this association leads to correct positioning of the catalytic domain on the membrane. Mutations in exon 5, which encodes

the phosphatase domain, as well as in exons 7 and 8, most of which determine premature stop codons, inactivate PTEN protein. By its lipid phosphatase activity, PTEN dephosphorylates PIP<sub>3</sub> and the phosphatidylinositol 3,4-bisphosphate (PIP<sub>2</sub>), determining the PI(4,5) bisphosphate and PI(4) phosphate formation, respectively. The opposite biochemical reaction is catalyzed by PI3K, which is associated with cell growth and cell survival. Thus, PTEN, which counteracts PI3K activity, is involved in cell death or modulation of arrest signal. PTEN prevents AKT phosphorylation, maintaining it in its inactive form, by dephosphorylation of PIP<sub>3</sub> and PIP<sub>2</sub>, leading to block cell growth. Such an ability seems to be mediated by cell-cycle arrest at the G1 phase and by increasing apoptosis (220).

In addition to lipid phosphatase activity, the PTEN catalytic domain can dephosphorylate proteins, one of which is represented by the focal adhesion kinase leading to inhibition of cell migration, invasion, and focal adhesion (220).

PTEN is regulated by TP53 (228) and TGF- $\beta$  (221); collaborates with INK4a/ARF (229) and MAGI-2 (220); and causes increased expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) (230), and c-myc (231).

It has been demonstrated that PTEN inactivation is associated with poor prognosis in conventional (clear-cell) renal cell carcinoma (232); in hepatitis C virus-positive cirrhotic hepatocellular carcinoma (230); in cervical cancer (233); and in tongue cancer (234). PTEN alterations are uncommon in head-and-neck squamous cell carcinoma (HNSCC) (235); breast cancer (236); and prostate cancer, melanoma, and glioma (224). *PTEN* mutations located outside exons 5, 6, and 7 represent a positive prognostic indicator for survival in endometrial carcinoma (222).

## Akt

The *Akt* family genes, which belong to a subfamily of protein kinases named AGC protein kinases (that encompasses protein kinase A [PKA] and PKC), encode for three Ser/Thr kinases and represent a major effector mediating survival signals. The three isoforms of Akt proteins are Akt1, Akt2, and Akt3 (also named PKB- $\alpha$ , PKB- $\beta$ , and PKC- $\gamma$ , respectively). *Akt1* is located on 14q32, *Akt2* on 19q13.1-2, and *Akt3* on 1q44. Overall, the three isoforms share >80% homology with each other (237). The three isoforms of the Akt family are ubiquitously expressed in mammals, but the levels of expression of the single member depends on the tissue analyzed, suggesting distinct roles (238).

Each Akt isoform possesses a pleckstrin-homology (PH) domain of approx 100 amino acids at the N-terminal position linked through a short Gly-rich region to the Ser-Thr kinase catalytic domain. The C-terminal region has the hydrophobic regulatory region (237). The role of PH domain is essential to bind PIP<sub>3</sub> and PIP<sub>2</sub>, to trigger Akt to the plasma membrane, and to induce a favorable conformation of Akt (237,239). Akt possesses six sites of phosphorylation: Ser124 and Thr450 are basally phosphorylated; Tyr315 and Tyr316 depend on Src; Thr308 represents the major site of regulation and is phosphorylated by 3-phosphoinositides-dependent protein kinase 1; and Ser473 is only required for maximal Akt activity, but the mechanism by which it is phosphorylated remains controversial (237).

Akt is phosphorylated and activated after cell stimulation from different growth factors and from a series of interleukins (reviewed in ref. 240). Furthermore, Akt is regulated by the calcium/calmodulin-dependent kinase kinase (CaMKK) and from protein phosphatases, such as PP2A, which dephosphorylate Akt and maintain it in an active

state (237), or PTEN, which dephosphorylates  $\text{PIP}_3$  and  $\text{PIP}_2$  and counteracts the action of PI3K. Once activated, Akt dissociates from the plasma membrane and translocates to both the cytoplasm and the nucleus, where it can phosphorylate a series of substrates carrying the consensus site RXXXS/T (239,240).

Akt inhibits programmed cell death directly through phosphorylation of Bad and caspase-9 and indirectly by inducing *de novo* gene expression of IKK protein kinase and transcription factors such as CREB and Forkhead (237,240). Akt determines cell survival also by virtue of its involvement in cell-cycle progression. In fact, Akt induces cyclin D expression and stabilization (239) and phosphorylates  $\text{p21}^{\text{CIP1}}$ ,  $\text{p27}^{\text{KIP1}}$  (237), and Mdm2 (238). Akt plays an important role in the regulation of metabolism by phosphorylating GSK-3 $\beta$  (237).

The levels of expression or of the activity of Akt protein can be evaluated by IHC, Western blot, immunoprecipitation, immunofluorescence, RT-PCR, real-time PCR, transfection assay, in vitro kinase assay, and electrophoretic mobility shift assay.

Analyses of tissue specimens showed that the protein encoded by Akt3 is overexpressed in poorly differentiated breast and prostate cancers. Akt1 is especially involved in the pathogenesis of sporadic thyroid cancer, whereas Akt2 seems to be the isoform that plays a pivotal role in several human tumors, such as ovarian (241), thyroid (242), and colorectal cancer (243). In addition to overexpression of the protein products, the constitutive activation of the three isoforms is required for oncogenic transformation. Consistently, Akt1 is constitutively phosphorylated in prostate, breast, and ovarian cancers (244). Akt can directly activate estrogen receptors (237), androgen receptor (237), and RT catalytic subunit of the human telomerase (237). Akt is regulated by EGFR (245), Raf pathway (246), JAK-1 (247), lysophosphatidic acid, and sphingosine-1-phosphate. Akt alterations are related to worse prognosis in colorectal (248), ovarian (249), breast (250), prostate (244), and pancreatic (251) cancers.

Deregulation of Akt expression appears to be involved in drug response and resistance in several tumors (252). Researchers continue to develop new drugs that can selectively block Akt activity through binding to the PH domain (239). Preclinical and clinical data suggest that the combination of two drugs at lower concentrations, such as taxol and a Raf-MAPK (MEK) inhibitor (237) or tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and a PI3K inhibitor (253), may achieve the block of Akt.

## COX-2

COXs (or prostaglandin H [PGH] synthase) are enzymes that catalyze the conversion of arachidonic acid to  $\text{PGH}_2$ . In mammals, cyclooxygenases are represented by two isozymes, encoded by two genes: *COX-1* (EC 1.14.99.1) and *COX-2*.

*COX-2* sequence, located on chromosome 1, shares 60% homology and catalytic activity with *COX-1*. At variance with *COX-1*, *COX-2* is not expressed in most tissues, but it can be rapidly induced by a wide number of extracellular and intracellular stimuli (254). A putative Tcf-4 binding element was identified in the *COX-2* promoter, supporting the idea that *COX-2* may be a downstream target of the APC- $\beta$ -catenin-Tcf-4 pathway (255). Assessment of *COX-2* status can be accomplished by Northern blot analysis, RT-PCR, *in situ* hybridization, Western blot, or IHC.

The pathogenic role of *COX-2* overexpression was first inferred in colon cancer. In 1995, it was demonstrated that a knockout mouse carrying a truncating mutation in the *APC* gene (*Apc* $\Delta^{716}$ ) had developed numerous polyps in the intestinal tract, a model for

FAP. When *COX-2* gene was deleted in the *Apc* $\Delta^{716}$  mouse, the number and the size of intestinal polyps were reduced (256).

Analysis of surgical specimens has shown that *COX-2* mRNA was more expressed in adenomas than in normal tissue and that this expression increased progressively with adenoma enlargement. Furthermore, *COX-2* overexpression cooperates with K-Ras in the progression of colorectal adenomas (257), and with vascular endothelial growth factor in tumor angiogenesis, and causes increasing expression of the antiapoptotic gene *BCL2* (258).

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin production. Since *COX-1* is involved in GI protection and *COX-2* is rapidly induced by several stimuli, current attempts are to synthesize new *COX* inhibitors able to bind and then block the *COX-2* activity in a selective way (259). Unfortunately, the mechanisms of action of NSAIDs are not fully understood. Nonselective (aspirin and sulindac) and selective (celecoxib and rofecoxib) NSAIDs are able to induce apoptosis by *COX*-dependent and *COX*-independent mechanisms. The mechanisms by which the inhibition of *COX-2* leads to a *COX*-dependent apoptosis are altered prostaglandin production; decreased angiogenetic factors; and increased concentrations of arachidonic acid that stimulate the conversion of sphingomyelin to ceramide, which is a mediator of apoptosis (259). Inhibitors of *COX-2* can exert their antiapoptotic effects by *COX*-independent mechanisms, such as inhibition of the activation of NF- $\kappa$ B, interference with the binding of peroxisome proliferator-activated receptor  $\Delta$  (PPAR $\Delta$ ) to DNA, and other *COX*-independent mechanisms not characterized (259).

Not all colorectal cancers analyzed showed *COX-2* overexpression. Low or absent *COX-2* expression is common among patients with colorectal cancer in families with hereditary nonpolyposis colon cancer (HNPCC) (260). Moreover, *COX-2* deregulation can be achieved through methylation of a region upstream of exon 1. Aberrant methylation was, in fact, detected in 13% of colorectal adenomas. These findings could lead to a limitation in the use of NSAIDs for chemoprevention, but other studies are needed to clarify this issue. A plethora of reports has shown that *COX-2* is overexpressed in a wide number of tumors (261).

*COX-2* overexpression correlates with poor prognosis and more aggressive disease in colorectal cancer (262), and breast cancer (263); NSCLC (264); gliomas (265); malignant mesothelioma (266); gastric carcinoma (267); cervical cancer in which *COX-2* overexpression also predicts the resistance to chemotherapy (268); ovarian carcinoma (269); and adenocarcinoma arising from Barrett's esophagus (270). These data suggest that the use of NSAIDs, especially those displaying selective inhibition of *COX-2*, could have a role in the prevention and treatment of a large number of malignancies.

## Chromosome 18q

The *DCC* (deleted in colon cancer) gene encodes for a transmembrane protein of approx 190 kDa, the netrin-1 receptor, belonging to the family of cell adhesion molecules. The *DCC* protein drives the migration of neuronal axons (271) and has a significant homology with neural cell adhesion molecule (NCAM) and other members of the *Ig* gene superfamily. Hence, the *DCC* gene product may be involved in the modulation of normal cell-cell and cell-matrix interactions. Furthermore, *DCC* protein induces apoptosis by proteolysis of specific receptors (272). The *DCC* gene spans approx 1.35 Mb in the chromosome 18q and comprises 29 exons. The expression of *DCC* has been evaluated by IHC, direct sequencing, and mRNA analysis.



Reduced expression of DCC has been seen in breast (273), ductal pancreatic (274), and prostatic carcinomas (275). Colorectal cancer is the most investigated model of the *DCC* gene. Decreased mRNA expression or aberrantly sized DCC transcripts have been shown in a number of primary colorectal cancers (276), while somatic mutations were identified in a restricted number of colorectal adenocarcinomas (277). Several reports suggested that DCC is involved in late stages of carcinogenesis of colorectal cancer (278) and may increase the metastatic potential of cancer cells (279). Consistently, *DCC* null immunophenotype has been proposed as a prognostic marker in patients at risk of liver metastases (280), and if carrying DCC protein-negative primary colorectal cancers (277) at stage II, they may benefit from adjuvant chemotherapy (levamisole and 5-FU).

Allelic imbalance analyses of chromosome 18q did not mirror the immunophenotypic findings (281,282). This discrepancy seems to be due because two other TSGs that cooperate with DCC in the colorectal carcinogenesis are located in the region 18q21-22. These two genes are *SMAD4* (previously named *DPC4*, as deleted in pancreatic cancer locus 4, or *MADR4*, as mothers against decapentaplegic related 4) and *SMAD2* (previously named *MADR-2* or *JV18*, as juvenile polyposis). *SMAD4* encodes for a nuclear transcription factor involved in TGF- $\beta$ 1 signaling and in angiogenesis (283). *SMAD2* interacts with the SKI protein and is involved in endodermal differentiation (283).

### **Genetic Markers Derived from DNA and Chromosomal Instability**

The term *chromosomal instability* refers to a wide spectrum of alterations that occur at either the DNA or chromosomal level. At the DNA level, chromosomal instability encompasses MSI and LOH, whereas at the chromosomal level, instability is restricted to LOH. MSI and LOH represent two unrelated phenomena that share a common analysis tool: microsatellite sequences.

Microsatellites, also known as SSR, are short genomic sequences that usually are present in the human genome as mono- to esanucleotides, repeated  $n$  times in tandem in large clusters. Their presence has been demonstrated in all human chromosomes with a frequency proportional to their dimension. Specific localization of microsatellites is mostly extragenic, but intragenic sequences have been described (284). Tandem clusters tend to be highly polymorphic in terms of size: wide variations among individuals suggest their use as polymorphic markers in characterizing individual genomes. Consistent with their simple sequence and condensed structure, microsatellite DNA is not transcribed or translated.

Errors occurring in microsatellite sequences during DNA replication produce expanded or shortened repetitive sequence units within the microsatellite and lead to the appearance of additional bands in otherwise normal alleles. This phenomenon (MSI) often, but not always, occurs in conjunction with mutations in MMR genes that are involved in DNA repair pathways. It has been proposed that MMR genes represent a third class of genes in addition to TSGs and oncogenes that could promote oncogenesis, if altered.

Confusion surrounds the terminology used to define MSI. Acronyms such as MSI, MIN, and MI refer to instability detected within microsatellite sequences. Moreover, no consensus is found in the literature regarding the number of markers necessary to classify a specimen as positive or negative for MSI, or regarding an unequivocal definition of MSI and replication error repair (RER). As to the first issue, several investigators proposed a minimum range of three to seven loci analyzed. For definitions of MSI

and RER, it has been suggested that the presence of MSI in at least one locus may be termed MSI positive or low-frequency MSI, while MSI detected in more loci tested, or in a generalized fashion, should be defined as RER-positive phenotype or high-frequency MSI. Biologically, RER-positive phenotype definitely contributes to the development of cancer, whereas MSI could lead to cellular death or proliferation advantage followed by selection of clones prone to malignant transformation. In addition, a cutoff of 29% has been proposed for the frequency of MSI, to discriminate between MSI and RER. Unfortunately, this cutoff cannot be applied to all types of tumors. It has been proposed that studies of sequences contained within cancer-associated genes be preferred for their better result accuracy rather than screening of markers outside such genes. Another unsolved issue concerns the choice of nucleotide repeats: dinucleotide microsatellites should be preferred over trinucleotides or tetranucleotides for monitoring genomic instability.

At variance with MSI, LOH has been shown to reflect loss-of-function mutations in the retained allele of a TSG within the affected chromosomal region (285). The two-hit hypothesis of oncogenesis postulated by Knudson (286) indicates a first inactivation of one allele of a TSG by mutations ranging from alteration of a single amino acid to a large gene deletion, with the second allele often inactivated by a less-precise pattern. The aforementioned model was first applied to hereditary tumors, in which the first hit represents a germline mutation and the second hit is the LOH that occurred at a somatic level and that affected the wild-type allele. It is possible that the two-hit hypothesis can be applied to sporadic tumors also, except that the two aforementioned events occurred at a somatic level. Detection of LOH relies on cellular economy for its implication in tumor transformation and progression, and it acquires meaning as an early diagnostic marker and as a prognostic and therapeutic response marker.

The clinical value of such markers in early diagnosis and their usefulness in the definition of prognosis and therapeutic response in neoplastic diseases is summarized. The data show a clustering of such markers within organs/sites/systems and, in particular for prognosis, a correlation with several clinicopathologic parameters (i.e., histotype, localization, timing progression, multifocality, and familiarity) that may serve to better define the disease outcome. Finally, the unresponsiveness to alkylating agents shown by MSI-positive patients makes such a marker a valid tool for evaluation of chemotherapy response.

Detection of microsatellite alterations in pathologic tissue requires a comparison with normal tissue from the same patient. Microsatellite analysis (LOH and MSI) is an easy, fast test. The relative small size of microsatellite sequences (100–300 bp) allows analysis on DNA specimens extracted from fresh or frozen tissue and from formalin-fixed and sometimes bouin-fixed specimens (in the latter case, nested PCR is required). Common methods for detecting MSI and LOH are radioactive PCR or Southern blot (LOH only) and denaturing acrylamide gel electrophoresis and fluorochrome PCR with an automatic sequencer. The latter approach allows a higher grade of analysis standardization and an objective interpretation of the results that no longer depends on the expertise of the operator.

### **MSI, LOH, and Cancer**

MSI and LOH have been observed in sporadic tumors of the colon, stomach, pancreas, bladder, hematopoietic system, lung, ovary, breast, endometrium, prostate, brain,

head and neck, and skin. Both types of alterations show loci clustering by tumor type, site, and body system. Consistent with a role in tumor initiation, such molecular alterations appear to occur early in the tumor development and may be detected at a preneoplastic stage, as well as in nonmalignant tissue. This finding suggests that, at least in some cases, such a pattern is compatible with a normal phenotype (287). MSI and LOH reportedly are useful in the evaluation of prognosis and therapeutic response in different tumors. As noted above for prognosis, a wide spectrum of positive or negative correlations of these markers with traditional clinicopathologic parameters (i.e., histotype, localization, timing of progression, multifocality, and familiarity) has proved partly useful to better define the disease outcome. MSI could offer a valid tool for evaluating responsiveness to chemotherapy because most cancer cells that resist alkylating agents exhibit the MSI phenotype (Table 1).

### ***Genetic Markers Derived from Nonrandom Chromosomal Abnormalities***

Recurring and highly consistent chromosomal aberrations have led to the identification of new protooncogenes at or spanning chromosomal breakpoints. Studies have shown that these genes are oncogenic and confirmed the pivotal role of chromosomal aberrations in tumor development. Specific translocations initially have been identified in hematologic tumors, and, subsequently, they also have been shown in a subset of solid neoplasms. In hematopoietic tumors, chromosomal translocations have two consequences: the juxtaposition of a protooncogene to the gene for a T-cell receptor (TCR) or an Ig protein, inducing oncogenic activation, and creation of a fusion gene encoding a chimeric protein. The genes involved often encode transcription factors, suggesting that disruption of transcriptional control plays a major role in oncogenesis. The main clinical application of these nonrandom chromosomal abnormalities is the diagnostic definition of several morphologically equivocal tumors followed by the assessment of minimal residual disease and therapeutic response. Because of the large number of chromosomal translocations identified in hematopoietic tumors, this chapter describes in detail only those with significant clinical relevance. Other translocations reported in hematopoietic tumors are provided in Tables 2 and 3.

### ***Hematopoietic Tumors:***

#### ***Protooncogene Activation and Tumor Suppressor Inactivation***

##### ***BCL2***

*BCL2* was one of the first oncogenes shown to be involved in nonrandom chromosomal translocations. It usually is rearranged with Ig heavy- and light-chain genes on chromosomes 14q32 (IgH), 2p11, and 22q11. In t(14;18)(q32;q21), approx 70% of the breakpoints on chromosome 18 cluster within a major breakpoint region (MBR) in the untranslated region of exon 3, and 20% occur in the minor cluster region (MCR) 20 kb downstream of *BCL2*; a few breakpoints cluster in the variant cluster region, 1.5 kb upstream or within the first noncoding exon and involve Ig light-chain variant translocation. Using new PCR techniques, new clusters between MBR and mcr, referred to as 3' *BCL2*, 5' mcr, and intermediate cluster region (icr), have been identified (288). As a consequence of the breakpoint locations, the protein coding domain of *BCL2* is maintained during translocation. Gene rearrangement results in overexpression of intact *BCL2* protein under the control of Ig-enhancer sequences.

**Table 1**  
**MSI and LOH in Cancer**

Evaluation of MSI and LOH in neoplastic diseases that cannot yet be considered of clinical relevance has been reported for ovarian and breast carcinomas and melanocytic lesions. A possible role of these markers in disease progression, poor outcome, and risk of malignant transformation has been suggested for these and other cancers.

### **Gastric Cancer**

- RER+ detection ranges from 15–30% (462), without any correlation with MMR gene mutations.
- RER+ (scored with 3 or more loci involved) is associated with antral location, intestinal subtype, both early and advanced stages, and nodal positive cases (463).
- RER+ (scored as more than 6 loci involved) is not restricted to patients with familial disease background and is significantly associated with female sex, lower tumor stage, and tumor localization in the distal part of the stomach (464).
- RER+ as a diagnostic or prognostic marker outside the context of HNPCC cases is currently of limited clinical value (287).
- Correlation of RER+ with tumor stage, localization, and histologic subtype seems to be a promising field.

### **Bladder Cancer (data concerning TCC)**

- The 60 tri- and tetranucleotide markers were screened for 50 TCC urine samples, obtaining at least one marker alteration, namely MSI and/or LOH, in 80% of cases analyzed (465).
- Data were confirmed by using a microsatellite panel comprehensive of 20 markers (466).
- Candidate TSGs involved in bladder cancer mapped at chromosome 9 are *p16*, *GAS1*, and *PTC* genes, but none showed alterations (467).
- LOH at 8p and 10q, namely 10q24.1–q24.3 and 10q26.1–26.2 bands (468,469), have been proposed as adverse prognostic markers; 8p deletion has been found to correlate with high-grade tumors (470), but putative TSGs as *POLB* and *PPP2CB*, mapped at the 8p region, did not reveal any mutations (471).
- Loss of function of TSGs as *DEL27*, *APC*, *DCC*, *TP53*, and *Rb* have been proposed as adverse prognostic markers; LOH at *DEL27* locus seems to be involved in cases showing an aggressive behavior (472).
- Consistent with their role in the regulation of the cell cycle, LOH of *TP53* and *Rb* has been mainly observed in high-grade and/or wide-invasive tumors (473), and associated with disease progression and survival reduction, especially if both markers are simultaneously altered.
- Taken individually, *Rb* correlates with increased mitotic index, whereas *TP53* seems useful in identifying chemotherapy-unresponsive patients (474).
- Generalized LOH and MSI may discriminate for the presence or absence of tumor cells in urine cytologic samples. LOH at 8p, 10q, *DEL27*, *Rb*, and *TP53* loci seems to correlate with poor outcome and chemotherapy unresponsiveness. LOH on chromosome 9 appears to correlate with both early and late events and its role needs to be further investigated.

### **Head-and-Neck Cancer (data concerning HNSCC)**

- HNSCC develops from accumulations of genetic events sometimes specific for early or advanced stage of the disease.

*continued*

**Table 1 (continued)**  
**MSI and LOH in Cancer**

**Head-and-Neck Cancer (data concerning HNSCC) (continued)**

- Losses of 9p21 and 3p14 regions are frequently detected in oral premalignant lesions (475,476), although mutations occurring at *p16* and *VHL* genes mapped within these regions have never been found.
- Correlation between generalized LOH and histology high-grade as well as advanced tumor has been reported, and evidence suggests that chromosome 18, more than others, is involved in short survival. In this view, at least three minimal deleted regions of chromosome 18, encompassing the q12, q21.1, and q21.2 bands, were frequently found lost in more aggressive cases (477,478).
- Candidate TSGs on 18q are *DCC*, *DPC4*, and *MADR2*. *DCC* gene, located within one of the three aforementioned minimal regions of deletion, is frequently found in a homozygous deletion status in HNSCC cell lines, but it is still controversial its involvement in cancerogenesis and progression of human HNSCC.
- Evidence suggests that simultaneous LOH at *TP53* and *Rb* loci is associated with poor survival and that these markers may be usefully applied for prognosis (479).
- LOH at 18q and 8p loci seems to correlate with tumor aggressiveness (480,481).

**Prostate Cancer**

- Positive correlation between frequency of LOH at specific loci and both tumor highgrade and cancer death has been reported (3.6% grade I vs 12% grade II) (482,483).
- LOH detection at 10q, 18q, 16q, and 11p chromosomal regions has been found to be associated with high histologic grade and recurrence (484).
- Although frequently referenced, LOH at the 18q21 region and progression of prostate carcinoma and TSGs possibly involved remain undetected (485,486).
- The 16q13–q24.3 region, and in particular the 16q24.1–q24.2 band, have been frequently found associated with aggressive disease (i.e., histologic high-grade and metastasis development but no mutations have been found occurring at *CDH1*, *HPR* CBFb TSGs located here) (487,488).
- A high rate of LOH (70%) at 11p region, harboring the *KAI1* gene, has been observed in metastatic foci from human prostate cancer, but further investigation is required (489).
- LOH at 10q, 11p, 16q, and 18q chromosomal regions as well as the frequency of generalized LOH correlates with adverse prognostic parameters such as high tumor grade, recurrence of neoplasia, advanced stage, and cancer death; 16q LOH could be a promising marker to identify patients who do not benefit from androgen therapy.

**Renal Cell Carcinoma**

- Validation has been provided for the microsatellite markers panel useful for the differential diagnosis in RCC subtypes (i.e., nonpapillary RCC, papillary RCC, chromophobe RCC, renal oncocytoma, and collecting duct carcinoma) (490).

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HNPCC, hereditary nonpolyposis colon cancer; HNSCC, head-and-neck squamous cell carcinoma; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; NSCLC, non-small cell lung cancer; Rb, retinoblastoma protein; RCC, renal cell carcinoma; RER, replication error repair; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer; TCC, transitional cell carcinoma; TSG, tumor-suppressor gene.

*continued*

**Table 1 (continued)**  
**MSI and LOH in Cancer**

**Renal Cell Carcinoma (continued)**

- Nonpapillary RCC is marked by LOH at chromosome 3p (98%), 8p (33%), 9 (33%), 14q (48%), and duplication of chromosome 5q (70%); papillary RCC shows trisomies of chromosome 3q (35%), 7 (80%), 8 (20%), 12 (35%), 16 (60%), 17 (90%), and 20 (30%); chromophobe RCC is characterized by a combination of LOH at chromosome 1 (100%), 2 (95%), 6 (88%), 10 (88%), 13 (95%), 17 (76%), and 21 (70%).
- A positive association has been found among trisomies 16, 12, 20, and partial loss of chromosome 14 and clinically more aggressive RCCs (491); 14q deletion positively associates with increased tumor grade and stage, demonstrating its validity as a marker in prognosis evaluation.
- Microsatellite markers located at chromosomes 1, 2, and 3 can be used for differential diagnosis of RCC; LOH at 14q may be usefully applied as an adverse prognostic marker overall, whereas LOH at 8p and 9p correlates with high stage in a subset of RCCs.

**Lung Cancer**

- Morphologic steps leading to lung neoplasia including, at least for SCC, hyperplasia, metaplasia, dysplasia, carcinoma *in situ*, and invasive and metastatic carcinoma, reflect the typical multistep process of carcinogenesis.
- The molecular mechanism of lung carcinoma has focused on the role of inactivation of *K-ras* (492), *TP53* (493), *p16-CDKN2-MTS1* (494), and *FHIT* (495).
- Allelotype studies showed that LOH at 3p, 13q, and 17p are involved in carcinogenesis of NSCLC, whereas deletions especially involving 2q, 9p, 18q, and 22q may play an important role in its progression (496).
- Both invasive carcinomas and the corresponding preneoplastic lesions were found widely affected by LOH at 3p; such a loss could be an early and crucial step in the pathogenesis of NSCLC and usefully applied as marker in the risk assessment (497).
- Preliminary data suggest that MSI at 2p and 3p regions (scored as 1 or more loci involved) may be detected at a high rate (69%) and may show a statistically significant correlation with poor prognosis; MSI at 2p and 3p loci may provide a useful prognostic marker in both stage I NSCLC and relapse risk assessment of operable forms (498,499).
- Frequency of MSI at 2p and 3p has proved to be an independent factor that could predict a decreased survival as well as familial clustering of malignancy, suggesting the presence of putative defects that might increase the sensitivity to a wide variety of environmental carcinogens (500).
- LOH occurring at chromosome 2 was found significantly associated with brain metastases, whereas specific 2q deletion seems to occur more frequently in moderately and poorly differentiated tumors than in well-differentiated ones (501).
- Recent data focused on the carcinogenic role of the 16q 24.1–24.2 band deletion not fitting with localization of *CDH1* suggest the existence of a different TSG involved in lung cancer progression (502).
- LOH occurring at two bands of the long arm of chromosomes 10, 10q21, and 10q23–25, never associated with *PTEN* gene mutations, has been proposed as an adverse prognostic marker in SCLC cases (503).
- The *p16* region seems to be the major target of deletion in primary NSCLC and preneoplastic lesions (504,505), whereas LOH at the same region has been reported to be present in a decreasing fashion in large cell carcinomas, SCC, and adenocarcinomas.

*continued*

**Table 1 (continued)**  
**MSI and LOH in Cancer**

**Lung Cancer (continued)**

- LOH at two distinct regions between D11S1758 and D11S12 and between HRAS and D11S1363, encompassing the 11p15.5 chromosomal portion, associated the former with tumor type and advanced stage, and the latter with cigarette consumption, female sex, and reduction of survival (506).
- LOH at 1p32/LMYC significantly correlates with regional nodal involvement as well as advanced clinical stages in NSCLC (507).
- The 3p and 9p losses seem to be related to the early stages and are useful in risk assessment; LOH spanning chromosome 2 is associated with poor differentiation and brain metastases; MSI occurring at 2p and 3p in addition to LOH at 1p32, 10q, 11p, and 16q may be associated with adverse prognosis and cigarette consumption (508).

**Colon Cancer**

- Clinical and pathologic stage are the only available prognostic parameters in colorectal cancer patients, but in a relevant percentage of cases, they failed to predict outcome or survival.
- Generalized LOH frequency was found equally distributed among the three types of colon and rectum cancers investigated (i.e., sporadic RER<sup>-</sup> cancers, sporadic RER<sup>+</sup> cancers, and cancers associated with ulcerative colitis); RER<sup>+</sup> phenotype seems to be associated with a survival advantage (509).
- LOH at 18q seems to correlate with adverse outcome; by contrast, RER<sup>+</sup> phenotype seems to contribute to better survival.

HNPCC, hereditary nonpolyposis colon cancer; HNSCC, head-and-neck squamous cell carcinoma; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; NSCLC, non-small cell lung cancer; Rb, retinoblastoma protein; RCC, renal cell carcinoma; RER, replication error repair; SCC, squamous cell carcinoma; SCLC, small-cell lung cancer; TCC, transitional cell carcinoma; TSG, tumor-suppressor gene.

The role of t(14;18) and BCL2 overexpression in tumorigenesis was demonstrated by *in vivo* studies. Transgenic mice bearing BCL2-*Ig* minigene harbor expanded B-cell compartments and developed follicular hyperplasia that eventually progressed to high-grade monoclonal lymphomas. When expression is directed to T cells, fully one third of the mice develop peripheral T-cell lymphomas. Long latency and progression from polyclonal hyperplasia to monoclonal malignancy are consistent with the hypothesis that oncogenic events in addition to BCL2 overexpression are necessary for tumor formation. Accordingly, in lymphomas arising in BCL2-*Ig* transgenic mice, a common second tumorigenic hit is translocation of the *C-MYC* oncogene.

Detection methods include Southern blot hybridization with probes specific for the MBR, MCR, and 5' regions of BCL2; PCR with primers specific for the MBR, mcr, and icr breakpoint regions (both on fresh and paraffin-embedded samples); long-distance PCR (LD-PCR); and FISH.

t(14;18) is the molecular hallmark of follicular lymphomas and is associated with 60–80% of these tumors. The translocation is found in 20% of DLCL cases, likely transformed from low-grade follicular lymphomas, and about 10% of HD cases. The t(2;18) and t(18;22) variant translocations have been described in 10% of B-chronic lymphocytic leukemia (B-CLL). Combined with morphologic and clinical observations, the finding of t(14;18) in lymph node aspirate may help define a differential diagnosis of follicular lymphoma (289).

**Table 2**  
**Nonfusion Genes in Hematopoietic Tumors**

Type	Translocation	Affected gene	Rearranged gene	Disease
Basic helix-loop-helix	t(7;19)(q35;p13)	<i>LYL1</i>	<i>TCR-β</i>	T-ALL
	t(7;9)(q35;q34)	<i>TAL2</i>	<i>TCR-β</i>	T-ALL
Cysteine-rich (LIM) proteins	t(11;14)(p15;q11)	<i>LMO1</i>	<i>TCR-δ</i>	T-ALL
	t(11;14)(p13;q11)	<i>LMO2</i>	<i>TCR-δ/α/β</i>	T-ALL
	t(7;11)(q35;p31)	<i>LMO2</i>	<i>TCR-δ/α/β</i>	T-ALL
Homeobox protein	t(10;14)(q24;q11)	<i>HOX11</i>	<i>TCR-α/β</i>	T-ALL
	t(7;10)(q35;q24)	<i>HOX11</i>	<i>TCR-α/β</i>	T-ALL
Others	t(10;14)(q24;q32)	<i>Iyt-10</i>	<i>IgH</i>	B-NHL
	t(14;19)(q32;q13)	<i>BCL-3</i>	<i>IgH</i>	B-CLL
	t(5;14)(q31;q32)	<i>IL-3</i>	<i>IgH</i>	Pre-B ALL
	t(7;9)(q34;q34)	<i>TAN1</i>	<i>TCR-β</i>	T-ALL
	t(1;17)(p34;q34)	<i>LCK</i>	<i>TCR-β</i>	T-ALL
	t(X;14)(q28;q11)	<i>C6.1B</i>	<i>TCR-α</i>	T-PLL
	t(14;15)(q32;q11-13)	<i>BCL8</i>	<i>IgH</i>	B-NHL
	t(2;7)(p12;q21)	<i>CDK6</i>	<i>IgK</i>	s-MZL
	t(7;14)(q32;q21)	<i>CDK6</i>	<i>IgH</i>	s-MZL
	t(1;14)(q21;q32)	<i>BCL9</i>	<i>IgH</i>	Pre-B ALL, B-NHL
	t(1;14)(q21;q32)	<i>MUC1</i>	<i>IgH</i>	B-NHL
	t(1;14)(q21;q32)	<i>MUM-2</i>	<i>IgH</i>	Myeloma, B-NHL
		<i>MUM-3</i>		
	t(11;14)(q23;q32)	<i>DDX6/RCK</i>	<i>IgH</i>	B-NHL
	t(12;22)(p13;q11)	<i>CCND2</i>	<i>IgL</i>	B-NHL
	t(12;14)(q24;q32)	<i>BCL7A</i>	<i>IgH</i>	B-NHL

TCR, T-cell receptor; IgH, immunoglobulin heavy chain; ALL, acute lymphoblastic leukemia (T-, B-, or pre-B-cell); B-NHL, B non-Hodgkin's lymphoma; B-CLL, B chronic lymphocytic leukemia; T-PLL, T prolymphocytic leukemia; LPL, lymphoplasmacytoid lymphoma; s-MZL, splenic mantle zone lymphoma.

t(14;18) is not exclusively associated with tumors. Using sensitive nested PCR, rare *BCL2*-JH-harboring cells have been seen in up to 50% of reactive tonsil and spleen and in peripheral blood of normal individuals, in whom the translocation frequency increases with age (290,291). Controversial data surround the prognostic impact of *BCL2* translocation. Studies have demonstrated that *BCL2*-rearranged and germline tumors undergo the same clinical behavior, and a negative prognostic marker is represented by *BCL2* protein overexpression (110,111). Moreover, no correlation between *BCL2* breakpoint location and either initial characteristics of the disease or survival of patients with follicular lymphoma could be demonstrated (292).

The presence of t(14;18) provides a useful genetic marker to monitor patients after therapy. The PCR persistence of residual *BCL2*-rearranged cells in the peripheral blood and bone marrow of patients in clinical remission identifies a group of people at high risk of relapse.



**Table 3**  
**Fusion Genes in Hematopoietic Tumors**

Translocation	Affected gene	Disease
t(1;19)(q23;p13)	<i>PBX1-E2A</i>	Pre-B ALL
t(17;19)(q22;p13)	<i>HLF-E2A</i>	Pro-B ALL
t(11;17)(q23;q21)	<i>PLZF-RARA</i>	APL
t(4;11)(q21;q23)	<i>AF4-MLL</i>	ALL/Pre-B ALL/ANLL
t(9;11)(q21;q23)	<i>AF9-MLL</i>	ALL/Pre-B ALL/ANLL
t(11;19)(q23;p13)	<i>MLL-ENL</i>	Pre-B ALL/T-ALL/ANLL
t(x;11)(q13;q23)	<i>AFX1-MLL</i>	T-ALL
t(1;11)(p32;q23)	<i>AF1P-MLL</i>	ALL
t(6;11)(q27;q23)	<i>AF6-MLL</i>	ALL
t(11;17)(q23;q21)	<i>MLL-AF17</i>	AML
t(3;21)(q26;q22)	<i>EVI-1-AML1</i>	CML
t(3;21)(q26;q22)	<i>EAP-AML1</i>	MDS
t(16;21)(p11;q22)	<i>FUS-ERG</i>	AML
t(6;9)(p23;q34)	<i>DEK-CAN</i>	AML
t(5;12)(q33;p13)	<i>PDGFβ-TEL</i>	CMML
ins(2;2)(p13;p11.2)	<i>REL-NRG</i>	B-NHL
inv(16)(p13;q22)	<i>CBFB-MYH11</i>	AML
t(3;5)(q25;q35)	<i>NPM-MLF1</i>	AML

ALL, acute lymphoblastic leukemia (T- or B-cell); AML, acute myelogenous leukemia; ANLL, acute nonlymphoblastic leukemia; APL, acute promyelocytic leukemia; CML, chronic myeloid leukemia; CMML, chronic myelomonocytic leukemia; MDS, myelodysplastic syndromes.

## **BCL1**

t(11;14)(q13;q32) translocation involves the *BCL1* locus on chromosome 11q13 and one of the joining regions of the IgH genes on chromosome 14q32, resulting in juxtaposition of *BCL1* with the IgH enhancer. Only sporadically variable (VH) genes or switch IgM (Sμ) may be involved (*163*). t(11;14) probably reflects an error in normal variable diversity joining (VDJ) recombination during normal precursor B-cell development. More than 80% of the breakpoints on chromosome 11 cluster in a 300-bp region known as MTC, centromeric to *BCL1* (*127*). An extension of >400 bp of the MTC has been identified in a patient with mantle cell lymphoma with t(11;14), suggesting that rare breakpoints in the MTC cluster can occur outside the previously defined region (*293*). Two MTCs have been identified (mTc1, 22 kb telomeric to *BCL1*, and mTc2, clustering in the 5' flanking region of *BCL1*) that are less frequently involved in translocation. Detection methods include conventional cytogenetics, Southern blot hybridization, PCR, FISH, and fiber FISH.

t(11;14)(q13;q32), the molecular hallmark of MCL, is detectable by Southern blotting and PCR, in up to 50% of these tumors (*294–296*). The finding that the percentage of *BCL1*-positive cases increases by FISH analysis (*297*) and the evidence that up to 90% of patients with mantle cell lymphoma overexpress BCL1 protein (*298,299*) suggest that deregulation of the *BCL1* gene occurs in many more cases than originally thought. No clinical differences were observed between *BCL1*-rearranged and germline mantle cell lymphoma, an indication that *BCL1* does not identify a clinically different lymphoma subset. Sporadically, t(11;14) has been found in lymphoid malignancies other than mantle cell lymphoma, including B-CLL and multiple myeloma (*300,301*).

In some cases, however, reclassification of these t(11;14)-bearing tumors included them in the mantle cell lymphoma histotype.

The protein kinases inhibitor flavopiridol has been used in clinical trials to inhibit a variety of protein kinases including cyclin D1 (302). Flavopiridol has been shown to induce growth arrest, cytotoxic cell death, and apoptotic changes in a variety of tumor types, including leukemia and lymphomas. In preclinical studies, the greatest activity of flavopiridol was observed in combination with chemotherapy agents (303). Because of its effect on cyclin D1, flavopiridol has been considered a potential agent for the treatment of patients with mantle cell lymphoma. In two clinical trials, minimal activity or partial responses and stable disease were observed, respectively. These controversial results possibly reflect schedule-dependent differences in efficacy. Similar observations have been made in CLL trials.

### c-myc

The translocations involving the *C-MYC* gene on chromosomes 8 and one of the Ig loci are of three types. Approximately 80% of cases involve translocation t(8;14)(q24;q32), which occurs between *C-MYC* and the genes for the Ig heavy chain. The remainder involve translocation between *C-MYC* and Ig light-chain sequences on chromosomes 2p11 and 22q11. In plasmacytomas, the breakpoints on chromosome 8 occur within the first noncoding intron of *C-MYC*, while in Burkitt's lymphoma, the translocations are more variable and occur in the 5' or 3' sequences flanking the gene or up to 300 kb upstream from the gene. Owing to the relocation of *C-MYC* near or within the strong transcription controls of the Ig gene, the translocation results in a loss of normal gene regulation and leads to constitutive *C-MYC* expression. Detection methods include Southern blot hybridization, LD-PCR, and FISH.

t(8;14)(q24;q32), and its variants t(8;22)(q24;q11) and t(2;8)(p11;q24), the molecular hallmark of Burkitt's lymphoma, are observed in almost all cases of the disease (304). These translocations occasionally can be detected in 15% of other intermediate to high-grade B-cell lymphomas, and sporadically in low-grade B-cell lymphomas. In sporadic Burkitt's lymphoma, translocation breakpoints cluster within the first exon or intron or immediately upstream from the gene, whereas in the endemic disease, translocations with breakpoints dispersed over approx 300 kb upstream from the gene are most frequent. Among other B-cell lymphomas, *C-MYC* rearrangement is observed in *BCL2*-positive follicular lymphomas undergoing high-grade transformation (305) and is considered a secondary genetic event involved in tumor progression.

### BCL6

Chromosomal translocations with the Ig gene regions are among the most common rearrangements involving chromosome 3q27. The *BCL6* gene can frequently rearrange with the *IgH* loci on chromosome 14 in t(3;14)(q27;q32), but occasional rearrangement with the *IgL* loci in t(3;22)(q27;q11) and t(2;3)(p11;q27) is observed. In t(2;3), the *BCL6* and *IgLκ* genes are juxtaposed in a head-to-head configuration. Rearrangements of *BCL6* with non-Ig genes have been described. Although many of the partner genes translocated with *BCL6* are unknown, some have been identified, including a novel H4 histone gene located on chromosome 6p21 (306), the B-cell transcriptional coactivator BOB1-OBFI on chromosome 11q23.1 (307,308), and the *TTF* gene that encodes a novel G protein on chromosome 4p11 (309). Many variant

rearrangements of *BCL6*, affecting chromosomes 1p32, 1p34, 3p14, 6q23, 12p13, 14q11, and 16p13, involve genes that have not been characterized (310). The gene for IL-21 receptor (*IL21R*) has been identified as the fusion partner with *BCL6* in t(3;16)(q27;p11) in DLCL (311). Detection methods for *BCL6* rearrangements include Southern blot hybridization and FISH with a cosmid spanning the 3q27 breakpoint region.

*BCL6* chromosomal translocations are associated with approx 50% of cases of DLCL and 10% of follicular lymphomas. In DLCL, *BCL6* rearrangement correlates with clinical presentation at extranodal sites, including the GI tract. Offit et al. (312) showed that patients with *BCL6* gene rearrangement had a favorable overall survival and survival without disease progression. This finding was questioned by another group (313), and a multivariate analysis has shown that rearrangement of 3q27, together with *BCL2* expression and the absence of a germinal-center phenotype, was associated with a poor prognosis in nodal DLCL (314). Moreover, within DLCL with a *BCL6* rearrangement, cases characterized by non-*Ig/BCL6* fusion have an overall survival significantly inferior to that of cases with *Ig/BCL6* fusion (315). The same group of investigators demonstrated that cell lines transfected with non-*Ig/BCL6* fusion genes expressed high levels of *BCL6* protein and showed characteristic punctuate nuclear staining, suggesting that non-*Ig/BCL6* translocation may play a pathogenetic role in a proportion of DLCL.

### **PAX5 t(9;14)(p13;q32)**

The *PAX* (for *paired homeobox*) 5 gene, mapping on chromosome 9p13, belongs to a transcription factor family that is involved in control of embryonic development and organogenesis. Members of this family contain two discrete DNA-binding domains (DBDs)—the paired box and the paired-type homeodomain—that display coordinate DNA-binding specificity. *PAX5* is normally expressed in fetal brain and liver during development but becomes restricted to B cells and testis after birth. In the B-cell lineage, *PAX5* undergoes downregulation during plasma cell differentiation. Knockout mice experiments have shown that *PAX5* is important for midbrain development and that its loss of function results in maturation arrest of lymphocytes at the pro-B-cell stage. By contrast, *PAX5* overexpression results in splenic B-cell proliferation. *PAX5* targets have been proposed to be CD19, B-cell receptor component  $Ig\alpha$  (mb-1), transcription factors N-MYC and LEF-1 (positively regulated by *PAX5*), and the p53 tumor suppressor (which is downregulated). B-cell SRC family tyrosine kinase BLK, which transforms lymphoid progenitors in an activated form but is dispensable for B-cell development and activation, is upregulated by *PAX5* (316). Furthermore, *PAX5* seems to be required for normal *IgH* VDJ recombination.

t(9;14)(p13;q32) results in juxtaposition of the *PAX5* gene with the *IgH* heavy-chain gene on chromosome 14 (317). The translocation is present in approx 50% of small lymphocytic lymphomas with plasmacytoid differentiation, the so-called lymphoplasmacytoid lymphomas (LPLs). These tumors possess a plasma cell-like phenotype with serum paraprotein production and have an indolent course followed by large-cell transformation. Deregulation of *PAX5* transcription by a translocated *IgH* promoter has also been described rarely in NHL subtypes other than LPL and in myeloma (318). An additional mechanism of *PAX5* activation seems to be gene mutation. Pasqualucci et al. (319) identified changes in the germline sequences of *PAX5* in most cases of DLCL. Other *PAX* genes are activated and have a role in oncogenesis.

## TAL1

*TAL1* (T-cell acute leukemia)-*SCL* (stem cell leukemia hematopoietic transcription factor) gene is located on chromosome 1p32. The encoded gene product is homologous to a number of proteins that are involved in the control of cell growth and differentiation. The region of homology is restricted to a 56 amino acid domain to form two amphipathic helices separated by an intervening loop. Such helix-loop-helix proteins are proposed to function as transcriptional regulatory factors based on their ability to bind in vitro to the E-box motif of eukaryotic transcriptional enhancers. The TAL1 protein may function as a transcriptional regulatory factor. Studies in mice indicate that TAL1 is essential for embryonic blood formation in vivo (320). In tissues, TAL1 is expressed in developing brain, normal bone marrow and mast cells, leukemic T-cells, and endothelial cells, but not in normal T cells (321). A recent report indicated an antiapoptotic effect of ectopic TAL1 expression in response to cytotoxic agents (322).

Tumor-specific alteration of TAL1 arises by either of two mechanisms. One mechanism is represented by t(1;14)(p32;q11), which transposes *TAL1* from its normal location on chromosome 1p32 into the TCR  $\alpha/\delta$  chain complex on chromosome 14q11. The second consists of a 90-kb deletion upstream of one allele of the *TAL1* locus, probably due to aberrant Ig recombinase activity that results in the fusion between *SCL-TAL1* and *SIL* (SCL interrupting locus, chromosome 1p33) (323,324). Both mechanisms disrupt the 5' end of the *TAL1* gene so that its expression is controlled by the regulatory elements of the *TCRA* or *SIL* genes that are normally expressed in T-cell ontogeny. The consequence may be an ectopic TAL1 production that activates a specific set of target genes that are normally silent. Breakpoints affecting the 3' side of TAL1 or occurring 25 kb downstream from the gene have been described (325).

Detection methods for t(1;14)(p32;q11) include conventional cytogenetics and Southern blot hybridization. Detection methods for deletions originating the TAL1-SIL fusion gene include Southern blot, DNA PCR, and RT-PCR.

Alteration of the *TAL1* gene is the most common genetic lesion known to be associated with T-cell ALL (T-ALL). Almost 25% of T-ALL patients exhibit *TAL1* deletions, and an additional 3% harbor the t(1;14) translocation. T-NHL or adult T-cell malignancies do not display *TAL1* aberrations. A study indicated that T-ALL patients with *TAL1* recombination had a significantly better outcome than other T-ALL patients without the recombination (326).

## BCL10 t(1;14)(p22;q32)

The *BCL10* gene maps on chromosome 1p22. It was cloned from a t(1;14)(p22;q32) translocation breakpoint from a patient with low-grade mucosa-associated lymphoid tissue (MALT) lymphoma. *BCL10* is composed of four exons within an approx 11.7-kb genomic segment. Its 2.8 kb transcript is expressed at relatively low levels in all normal tissues, with the highest expression levels in spleen, lymph node, testis, and developing CNS. The *BCL10* gene encodes a predicted protein of 233 amino acids, which contains an amino-terminal caspase recruitment domain (CARD) from residues 13 to 101 homologous to that found in several proteins involved in the regulation of apoptosis. Its C-terminal 132 amino acids contain no known motifs. In normal B-cells, the BCL10 protein is primarily located in the cytoplasm, while it becomes strongly expressed also in the nuclei of t(1;14)-bearing MALT lymphoma cells. BCL10 is a positive regulator of antigen receptor-mediated NF- $\kappa$ B activation through interaction

with MALT1 or the API2-MALT1 fusion product. Despite activation of NF- $\kappa$ B, *BCL10* acts as a tumor suppressor in vitro and promotes apoptosis by activating caspase-9.

The t(1;14)(p22;q32) translocation juxtaposes the entire coding region of *BCL10* to chromosome 14 under control of the Ig enhancer element. All *BCL10* breakpoints thus far characterized cluster within the 5' promoter region of the gene. The t(1;14) translocation is frequently associated with *BCL10* gene inactivating mutation. The regions more frequently mutated include the junction of exons 3 and 4 (with loss of a splice acceptor and deletions involving codons 116–121 or 116–126) and two polyA and polyT stretches (beginning at codon 43 and 165, respectively) at which deletions or insertions of one or two bases result in frameshifts. Frameshift mutations produce two kinds of *BCL10* truncation: CARD truncation and C-terminal truncation distal to the CARD (327). CARD truncation mutants lose apoptotic activity and fail to induce NF- $\kappa$ B, whereas mutants with C-terminal truncations retain NF- $\kappa$ B activation but do not induce apoptosis. It is then speculated that mutant *BCL10* would lose its proapoptotic function, conferring a growth advantage on tumor B-cells, and constitutive NF- $\kappa$ B activation could provide both antiapoptotic and proliferative signals by upregulating transcription of specific targets.

The methods to detect *BCL10* gene mutation include PCR-SSCP and direct sequencing starting from genomic DNA or cDNA, while the t(1;14) translocation can be detected by conventional karyotyping, Southern blot, and metaphase and interphase FISH (328).

*BCL10* gene alteration in tumors is a rare event. t(1;14)(p22;q32) translocation (or its variant t[1;2][p22;q22] involving the Ig- $\kappa$  light-chain locus) has been restricted to 5% of MALT lymphomas, as well as the *BCL10* gene mutation that has been found in 7–10% of these tumors (329). A slightly higher frequency of *BCL10* mutations in high-grade MALT lymphomas has been observed, suggesting that mutations may underlie histologic progression. Moreover, among low-grade MALT lymphomas, mutations were found in a subset of cases not responding to anti-*Helicobacter pylori* eradication therapy, but not in tumors that regressed completely; this observation may have important clinical implications. Data about other hematologic malignancies are controversial, and the rare involvement of *BCL10* in follicular lymphoma and DLCL (329–331) has not been confirmed by other studies, at both DNA and cDNA levels (332–334). Extensive work excluded a significant involvement of *BCL10* gene mutation in the pathogenesis of different solid cancers, including germ cell tumors (334), prostate carcinoma, SCLC, head-and-neck tumors, renal carcinoma, sarcomas (335), CLL, T-ALL (336), multiple myeloma, and plasma-cell leukemia (337). Therefore, *BCL10* gene alteration is a genetic event associated with a small percentage of the MALT subtype of NHL. If the preliminary clinical findings are confirmed in a larger controlled study, *BCL10* gene mutation could be used to identify prospectively those MALT patients who would not benefit from *H. pylori* therapy and for whom a chemotherapeutic approach should be followed.

## Hematopoietic Tumors: Chimeric Proteins

### NPM-ALK

The nucleolar phosphoprotein gene, nucleophosmin (*NPM*), is a highly conserved gene located on chromosome 5q35. Its protein product is involved in the late stages of ribosomal assembly and functions as a target for CDK2/cyclin E in the initiation of centrosome duplication. The *ALK* gene, a newly characterized gene located on chromosome 2p23, codes for a novel 200-kDa transmembrane protein kinase that belongs

to the insulin receptor subfamily. Whereas NPM is expressed ubiquitously at high levels, the normal expression of ALK is restricted to neural tissues and is important for normal neural development and function. It has been hypothesized that ALK may serve as a receptor for a yet unidentified neurotropic factor. In this context, in vitro studies showed high-affinity binding of ALK to pleiotrophin, a polypeptide growth factor that induces proliferation in a wide range of epithelial, endothelial, and mesenchymal cell lineages (338), and to its homologous midkine (339).

t(2;5)(p23;q35) generates a fusion *NPM-ALK* gene that encodes a chimeric protein. The NPM-ALK protein consists of N-terminal sequences derived from the *NPM* gene fused to C-terminal cytoplasmic sequences from the *ALK* gene, including the consensus protein tyrosine kinase residues. t(2;5) results in the transcription of ALK driven off the strong NPM promoter, leading to inappropriate expression and constitutive activation of a truncated 80-kDa ALK protein. Because of the breakpoint location, the fusion protein lacks extracellular and transmembrane domains and has intracellular localization. The NPM-ALK hybrid protein is thought to have a key role in tumorigenesis by aberrant phosphorylation of intracellular substrates.

Variant chromosomal translocations involving the *ALK* locus at chromosome 2p23 have been observed, and the *ALK* fusion partners identified (340) (Fig. 5). *TPM3-ALK* is expressed from a t(1;2)(q25;p23) translocation fusing the N-terminal 221 residues of *TPM3* to the cytoplasmic portion of *ALK*. *TPM3* is a nonmuscle tropomyosin fused with the truncated *NTRK1* receptor tyrosine kinase in papillary thyroid cancer. This protein contains an N-terminal coiled-coil structure that allows self-association and leads to activation of the *TPM3-ALK* fusion protein. The t(2,3)(p23;q21) translocation leads to the formation of three variant fusion proteins—*TFG-ALK<sub>S</sub>* (85 kDa), *TFG-ALK<sub>L</sub>* (97 kDa), and *TFG-ALK<sub>XL</sub>* (113 kDa) (341)—depending on the chromosomal breakpoint in the *TFG* gene (introns 3, 4, and 5, respectively). *TFG*, which stands for *TRK*-fused gene, was originally cloned as a fusion partner for *NTRK1* in papillary thyroid carcinoma (see discussion under Epithelial Tissue). As for *TPM3*, it contains a coiled-coil domain that can mediate the oligomerization and constitutive activation of the fused tyrosine kinase. The *ALK* fusion partner involved in the inv(2)(p23;q35), *ATIC*, encodes an enzyme responsible for the final steps of *de novo* purine nucleotide biosynthesis. The *ATIC-ALK* chimeric kinase is driven to homooligomerization and constitutive activation by the N-terminal 229 residues of *ATIC* fused to *ALK*. The *CLTC-ALK* variant fusion protein is expressed from the t(2;17)(p23;q23) translocation. *CLTC* represents the heavy chain of the clathrin molecule contained in the vesicles that transport molecules through the cellular compartments. The homodimerization and activation of the *CLTC-ALK* chimera results from the fact that, normally, clathrin molecules oligomerize and form trimolecular complexes. The t(2;19)(p23;q13.1) leads to the expression of a *TPM4-ALK* fusion protein, where *TPM4* is a nonmuscle tropomyosin highly related to *TPM3*. Additional *ALK* rearrangements have been cloned. *Moesin*, mapped on chromosome Xq11–12, has been identified as the partner for *ALK* in the *MSN-ALK* fusion (342). The hybrid *MSN-ALK* protein has a molecular weight of 125 kDa and contains an active tyrosine kinase domain. In contrast to other translocations involving the *ALK* gene, the *ALK* breakpoint in this case occurs within the intronic sequence coding for the juxtamembrane portion of *ALK*. In addition, *ALO17* (KIAA1618), a gene with unknown function, was fused to *ALK* in a case with t(2;17)(p23;q35); *CARS*, encoding the cysteinyl-tRNA synthetase, was fused to *ALK* in a case with t(2;11;2)(p23;p15;q31). In contrast to the NPM-ALK chimeric protein that

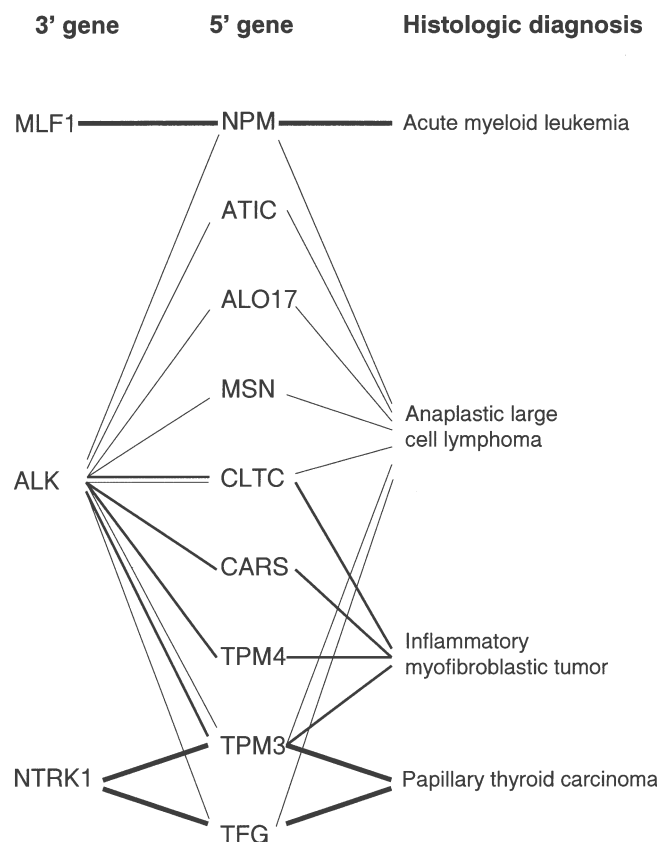


Fig. 5. *ALK* and *NTRK1* rearrangements. *ALK* gene may have more than one partner gene—some of which share with different pathologic entities (acute myeloid leukemia and papillary thyroid carcinoma)—leading to variant of the same histologic entity. Interestingly, among *ALK*-based different fusion genes, the same fusion type, *ALK-TPM3*, is shared by two different histologic entities (anaplastic large cell lymphoma and inflammatory myofibroblastic tumor).

has nuclear and cytoplasmic localization, the other variant *ALK* fusion products are localized only in the cytoplasm, with the exception of *MSN-ALK*, which exhibits a distinctive membrane-restricted pattern of *ALK* labeling. Since *ALK* protein is not expressed in adult normal tissues, the positivity and the staining pattern with *ALK*-specific antibodies are relevant in the diagnosis of some tumors.

The detection methods for *ALK* rearrangements are two-color FISH, RT-PCR, and IHC with MAb recognizing a formalin-resistant epitope in the chimeric and the 200-kDa normal human *ALK* proteins.

The presence of t(2;5) is specifically associated with 50–60% of CD30-positive anaplastic large cell lymphomas (ALCLs), which represent a subset of high-grade NHL. This marker identifies a subgroup of morphologically heterogeneous ALCLs with T/null phenotype that are characterized by a more favorable clinical course than *NPM-ALK*-negative ALCLs (343). ALCLs bearing alternative *ALK* rearrangements (10–20% of cases) are indistinguishable from ALCLs with classic t(2;5) (344). Some of the described rearrangements have been observed only in ALCL tumors, such as *NPM-ALK* itself; *ATIC-ALK*; and the three forms of *TFG-ALK*, *MSN-ALK*, and *ALO17-ALK*.

Others have been found in both ALCL and inflammatory myofibroblastic tumors, such as *TPM3-ALK* and *CLTC-ALK*, while *TPM4-ALK* and *CARS-ALK* have been seen to date only in inflammatory myofibroblastic tumors.

### **API2-MALT1 t(11;18)(q21;q21)**

The *API2* gene (also known as *c-IAP2*, *HIAP1*, or *MIHC*) maps on chromosome 11q21. It belongs to a family of inhibitors of apoptosis first identified in baculoviruses. It contains three copies of baculovirus inhibitor of apoptosis repeat (BIR) motifs, a middle CARD, and a C-terminal zinc-binding finger domain. The BIR domains are involved in inhibition of activated caspases (3, 7, and 9) through interaction with TNF-associated factor proteins. *MALT1* is localized on chromosome 18q21. It comprises an N-terminal death domain, which acts as a homotypic interaction module, followed by two Ig-like C2 domains and a caspase-like domain. MALT1 nucleotide sequence predicts an 813 amino acid protein that shows significant sequence similarity to the CD22 $\beta$  and laminin 5 $\alpha$ 3b subunit. MALT1 protein has been identified as a paracaspase (345), a caspase-like protease with altered substrate specificity compared with caspases. Through yeast two-hybrid system experiments, it has been demonstrated that MALT1 binds BCL10, the protein involved in the other MALT lymphoma translocation, by interacting with the two Ig-like domains (345). Under normal conditions, BCL10 and MALT1 form a tight complex that serves to oligomerize and activate the caspase-like domain of MALT1, leading to induction of NF- $\kappa$ B.

The t(11;18)(q21;q221) chromosomal translocation fuses the amino terminal of the *API2* gene product to the C-terminal of the *MALT1* gene product and generates a chimeric fusion product (346). All the breakpoints in the *API2* gene occur in introns downstream of the third BIR domain but upstream of the C-terminal zinc finger, with the highest frequency just before the CARD. Conversely, the breakpoints in the *MALT1* gene are more variable, occurring in four introns upstream of the caspase-like domain (346,347). As a result, the *API2-MALT1* fusion transcripts always comprise the N-terminal region of *API2* with the three intact BIR domains in frame with the C-terminal region of *MALT1* containing an intact caspase-like domain. Whereas full-length *API2* and *MALT1* do not significantly activate NF- $\kappa$ B, the fusion protein significantly increases NF- $\kappa$ B activation (345). The signaling pathway, which is maintained at basal level of expression in normal cells, can be perturbed by either t(1;14) or t(11;18) in MALT lymphoma, resulting in marked overexpression of BCL10 or *API2-MALT1* fusion proteins and in a dramatic increase in NF- $\kappa$ B activity, which is likely to be critical in lymphoma progression. A novel three-way variant translocation, t(11;12;18)(q21;q13;q21), has recently been described in a MALT lymphoma of the lung (348).

The methods to detect the standard t(11;18)(q21;q21) translocation include conventional cytogenetics; RT-PCR on fresh and formalin-fixed, paraffin-embedded archival tissue; and dual-color FISH in interphase nuclei and metaphase chromosomes on fresh and archival tumor tissue.

The t(11;18)(q21;q21) translocation is specifically associated with 30–40% of extranodal marginal zone B-NHL of MALT type (349,350), where it is usually the sole chromosomal aberration. This translocation is frequently found in advanced gastric MALT lymphomas, and almost exclusively in those that fail to respond to *H. pylori* eradication (351,352). Thus, detection of the translocation should help the clinical management of patients with tumors resistant to antibiotic therapy.



**BCR-ABL *t*(9;22)(*q*34;*q*11)**

The normal cellular *BCR* gene is located on chromosome 22q11.21. It spans a 135-kb region and contains 23 exons. The *BCR* gene is expressed as mRNA of 4.5 and 6.7 kb. It encodes a 160-kDa phosphoprotein associated with a Ser/Thr kinase activity and shows autophosphorylation activity as well as transphosphorylation activity for several protein substrates. The *c-ABL* gene, mapping on chromosome 9q34, is 225 kb in size and is expressed as either a 6- or 7-kb mRNA transcript. The *ABL* gene codes for a 145-kDa tyrosine kinase with nuclear localization. The DNA-binding activity of the ABL protein is regulated by CDC2-mediated phosphorylation, suggesting a cell-cycle function for *ABL*. The gene is implicated in processes of cell differentiation, cell division, cell adhesion, and stress response. The tyrosine kinase activity of nuclear ABL is regulated in the cell cycle through a specific interaction with Rb protein. ABL activity is negatively regulated by its SH3 domain through an unknown mechanism, and deletion of the SH3 domain turns ABL into an oncogene.

*t*(9;22)(*q*34;*q*11) translocation, which transposes the *ABL* gene from chromosome 9 to the center of the *BCR* gene on chromosome 22, results in a head-to-tail fusion of the two genes and the formation of the Philadelphia (Ph) chromosome. The 5' exon of the *ABL* gene lies at least 300 kb upstream from the remaining ABL exons, and the very long intron is the target for translocation. Although the position of the breakpoint on chromosome 9 varies considerably, the breakpoint on chromosome 22 is clustered in an area called bcr for breakpoint cluster region. The BCR-ABL-encoded product is a chimeric 210-kDa protein that has bcr information at its N-terminus and retains most of the normal ABL protein sequences. In some tumors, the *ABL* gene can be juxtaposed to the 5' region of the *BCR* gene in a *t*(9;22) translocation cytogenetically indistinguishable from the Ph chromosome. In these cases, a unique ABL-derived tyrosine kinase of 180 kDa is produced (353). The functional consequence of the BCR-ABL fusion is increased tyrosine kinase activity. Sequences within the first exon of BCR appear to be essential for this activation and probably work through direct physical binding to the kinase regulatory domain of ABL. Besides *BCR-ABL* rearrangement, point mutation in the *ABL* kinase domain and *BCR-ABL* amplification have been described in patients with advanced Ph+ leukemias (354), which are suggested to represent second mutational events during the course of CML.

Detection methods for *t*(9;22)(*q*34;*q*11) include conventional cytogenetics, Southern blot hybridization, two-color FISH, and RT-PCR. ABL kinase domain mutation can be detected by RT-PCR-RFLP and direct sequencing.

*t*(9;22) (Ph+) represents a diagnostic tumor-specific marker associated with more than 90% of CML that has an unfavorable evolution to AML or ALL. A striking correlation between the site of the breakpoint within the bcr on chromosome 22 and the length of time between presentation and onset of acute phase was demonstrated; the patients with 5' breakpoint had a four-fold longer chronic phase than those with a 3' breakpoint (355,356). Optimization of RT-PCR protocols has allowed monitoring of minimal residual disease in patients with Ph+ CML undergoing bone marrow transplantation (BMT). Several studies showed that PCR negativity indicates complete eradication of the leukemic clone, and PCR-positivity is associated with a relapse in T-depleted transplanted patients or those undergoing transplantation in the advanced phase. PCR-positive patients undergoing transplantation in the chronic phase or those receiving nonmanipulated bone marrow have a slightly higher risk of relapse than PCR-negative patients. In these cases it has been demonstrated by competitive PCR that a

low number of BCR-ABL transcript molecules are associated with prolonged complete remission, and patients with an increasing number of transcript molecules are subject to relapse (357,358).

The variant t(9;22), a translocation cytogenetically indistinguishable from that of CML but with a different breakpoint in the *BCR* gene, is found in approx 10% of patients with *de novo* ALL (359).

Inhibition of BCR-ABL tyrosine kinase activity has been introduced as a therapeutic approach in patients with CML. Administration of imatinib (STI-571) resulted in an antileukemic effect in CML patients in whom treatment with standard chemotherapy had failed (360). Primary refractoriness or relapse after imatinib treatment is observed in a significant proportion of patients in advanced-stage disease or in Ph+ ALL (354,361). This relapse has been associated with either *BCR-ABL* gene amplification or single amino acid substitutions affecting residues that are in direct contact with ATP or are within the ATP pocket of the kinase domain of ABL and resulting in structural changes that could influence inhibition sensitivity. Strategies for overcoming resistance have been suggested exploiting dependence of BCR-ABL protein on the molecular chaperone heat shock protein 90 (Hsp90). Drugs that inhibit Hsp90 (geldanamycin and its analog 17-AAG) have been shown to induce degradation of BCR-ABL and reduce cell growth in vitro; their clinical application in STI571-resistant patients has been suggested (362).

### **PML-RARA (t15;17)(q22;q12)**

The *PML* (promyelocytic leukemia) gene maps on chromosome 15q22. It codes for a DNA-binding zinc-finger protein with a potential leucine zipper motif. The *PML* protein is expressed at significantly high levels in G1-phase of the cell cycle and at a lower levels in S-, G2-, and M-phases. *PML* exhibits multiple biologic functions. It is a mediator of interferon function and immune surveillance and acts as a proapoptotic factor and as a tumor suppressor (363). *PML* upregulation by oncogenic *RAS* is involved in the control of *p53* acetylation (364). In mice, *PML* regulates hematopoietic differentiation and controls cell growth and tumorigenesis (365). *PML* function is essential for the tumor growth-suppressive activity of retinoic acid and for its ability to induce terminal myeloid differentiation of precursor cells. *PML* is needed for the retinoic acid-dependent transactivation of the *p21* (*WAF1-Cip1*) gene, which regulates cell-cycle progression and cellular differentiation (365).

The *RARA* gene maps on chromosome 17q12. It is homologous to the receptors for steroid and thyroid hormones and codes for a nuclear receptor protein that binds the retinoic acid ligand and DNA through a zinc-finger region, thereby presumably activating a set of target genes.

t(15;17)(q22;q12) is an important example of a transcription fusion factor, in which the *PML* gene on chromosome 17 is fused with the *RARA* gene on chromosome 15. In the chimeric gene, the promoter and first exon of the *RARA* gene are replaced by part of the *PML* gene. The *PML* breakpoints are clustered in two regions on either side of an alternative spliced exon. The translocation chromosome generates a PML-RARA chimeric transcript. Alternative splicing of PML exons produces multiple isoforms of the *PML-RARA* mRNA, even within a single patient. The *PML-RARA* fusion RNA encodes a predicted 106-kDa chimeric protein that contains most of the *PML* sequences fused to a large part of the *RARA* gene, including its DNA- and hormone-binding domains. The oncoprotein PML-RARA suppresses transcription by recruiting histone

deacetylase (HDAC) and rendering the nearby chromatin inaccessible to transcriptional activators (366). This results in interference with normal cell growth and differentiation. The ability of the chimeric *PML-RARA* gene to initiate tumorigenesis was demonstrated in transgenic mice, which exhibited a partial block of differentiation in the neutrophil lineage and eventually progressed to overt myeloid leukemia.

In addition to *PML*, *RARA* can form rare fusion proteins with four other genes: *promyelocytic leukemia zinc-finger* gene (*PLZF*) t(11;17)(q23;q21), which is a zinc-finger transcription factor expressed in immature hematopoietic cells and implicated in the development of the CNS (367); *nucleophosmin* gene (*NPM*), the same gene rearranged with *ALK* in ALCLs (368); *STAT5b* (369); and *nuclear mitotic apparatus* gene (*NuMA*) (370). Detection methods include conventional cytogenetics, Southern blot hybridization, FISH, and RT-PCR.

t(15;17)(q22;q21) is associated with approx 100% of cases of APL. The molecular characterization of *PML-RARA* has clinical prognostic impact. This genetic aberration represents a tumor-specific marker for a correct diagnosis of APL, and because its presence is related to a good response to all-*trans* retinoic acid (ATRA), it permits the use of a specific therapy based on the use of this retinoid, which acts by overcoming the block of maturation at the promyelocytic stage and inducing terminal differentiation into granulocytes. Treatment with ATRA in APL patients induces disease remission transiently, and relapse occurs in approx 30% of patients. This relapse can partly be explained by the occurrence of missense mutations in the LBD of *PML-RARA* that prevent interaction with ATRA (371). In these cases, resistance to the differentiating action of ATRA could be overcome by cotreatment with HDAC inhibitor sodium phenyl butyrate (372). HDAC inhibitors may also find a relevant clinical application in the treatment of *PLZF-RARA*-positive APLs that are less sensitive to the action of ATRA and have unfavorable prognosis compared with *PML-RARA* APL (373). In *PML-RARA* APL, it has been observed that the persistence of residual transcript during clinical remission allows identification of patients with high risk of relapse for whom further therapeutic treatment might be required (374).

### **AML-ETO t(8;21)(q22;q22)**

The *AML* gene, located on chromosome 21q22, is the human homolog of *Runt*, an important gene in *Drosophila* that regulates segmentation. Structural analysis of the *AML* gene showed that the 5' portion of the gene contains the *Runt* homologous sequences, a DBD, and dimerization sequences, whereas the 3' portion contains gene transactivation sequences (375). In adults, the *AML* gene is ubiquitously expressed in several tissues, particularly in bone marrow cells. Because *AML* knockout mice die during embryonic development, secondary to the complete absence of fetal liver-derived hematopoiesis, it is suggested that *AML*-regulated target genes are essential for definitive hematopoiesis of all lineages (376). The *ETO* gene maps on chromosome 8q22. It comprises 13 exons distributed over 87 kb of genomic DNA (377). *ETO* structurally belongs to the zinc-finger transcription factor genes. By Western blot analysis, the *ETO* product was identified as a 70-kDa protein associated with the nuclear matrix (378). Its biologic function is unknown. *ETO* is expressed in several tissues, mainly during fetal life, with the highest mRNA amounts occurring in brain and heart. *ETO* is specifically expressed in CD34<sup>+</sup> hematopoietic stem cells (379).

t(8;21)(q22;q22) leads to the fusion of the *AML* and *ETO* genes. The resulting fusion gene transcribes a hybrid mRNA and is translated into a 94-kDa AML-ETO

chimeric protein. The *AML-ETO* chimeric gene contains, in 5', *Runt* but not trans-activation sequences of AML; in 3', the gene contains the whole coding sequence of ETO, whose expression is regulated by the AML promoter (375). In vitro transfection experiments suggest that the AML-ETO fusion protein can suppress the normal AML protein function by inhibiting myeloid differentiation (380). Thus, the neoplastic transformation either may result by a dominant negative effect of the AML-ETO hybrid protein, which blocks the transcription of specific genes involved in myeloid differentiation, such as granulocyte macrophage CSF or, alternatively, may be promoted by aberrant ETO transcription under the effect of AML promoter. Detection methods include two-color FISH, RT-PCR, and Western blot.

t(8;21), the most frequent cytogenetic alteration observed in AML, is associated with 20% of AML M2 and is found in approx 5% of AML M1 by RT-PCR analysis (381). Except in rare pediatric cases, patients carrying this genetic abnormality usually have a favorable clinical course (382). In patients with complete clinical remission after conventional chemotherapy, and autologous or allogeneic BMT, the AML-ETO transcript is frequently found by RT-PCR (383,384). The biologic meaning of this finding is unknown. It is speculated that rare t(8;21)-positive cells persistent in the bone marrow of patients in clinical remission represent a clonal population only partially transformed and are not able to develop into overt leukemia. Owing to the constant persistence of genetically aberrant cells during remission, the minimal residual disease monitoring by RT-PCR does not seem to provide useful clinical and therapeutic information.

### **Other Translocations**

A number of other chromosomal translocations have been described in hematopoietic tumors, which either juxtapose protooncogenes to antigen-receptor genes or lead to the formation of fusion genes (385–387). These specific translocations are listed in Tables 2 and 3.

### **Solid Tumors**

Investigation of solid-tumor translocations has concentrated on sarcomas, whose cytogenetics have been well studied (385). In sarcomas, specific chromosomal translocations have been associated with distinct tumor histotypes, thus providing a clinical application in the differential diagnosis of sarcomas with difficult morphologic diagnosis assessment (e.g., PNET, synovial sarcoma, rhabdomyosarcoma) and, in some cases, a prognostic assessment. Moreover, these markers can be potentially used for monitoring minimal residual disease. The gene more frequently involved in these specific chromosomal translocations is *EWS*. Molecularly, the oncogenic conversion of *EWS* follows a common scheme of activation that exchanges its putative RNA-binding domain (RBD) with the DBDs of ETS family transcription factor genes (*FLI1*, *ERG*, *ETV1*, *E1AF*, *FEV*, *ZSG*) or other transcription factor genes (*ATF1*, *WT1*). This fusion may be necessary for the *EWS*-associated oncogenesis, and the transcription factor partner in the chimeric proteins may determine the specific tumor type. The fusion of a member of the ETS family of DNA-binding proteins (FL1, ERG, ETV1, E1AF, FEV, ZSG) with *EWS* produces pPNET, ATF1 with *EWS* to clear cell sarcoma, and WT1 with *EWS* to intraabdominal desmoplastic small round cell tumor. On the other hand, *FUS* and *EWS* proteins may functionally act as equivalents when fused with the transcription factor *CHOP* in myxoid liposarcoma (Fig. 6). These apparently opposite findings led to the hypothesis that *EWS* and *FUS* proteins may be interchangeable in the

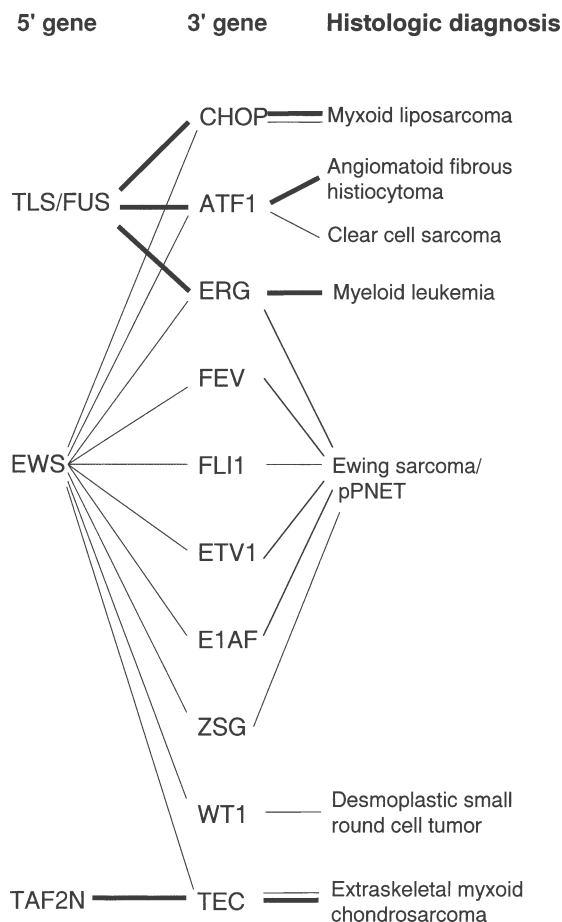


Fig. 6. *EWS* and *TLS/FUS* rearrangements. *EWS* gene may have more than one partner gene leading to different histologic entities. Fusion of *EWS* with one member of the ETS family of DNA binding proteins (*ERG*, *FEV*, *FLI1*, *ETV1*, *E1AF*, *ZSG*) gives rise to pPNETs and with *WT1* to desmoplastic small round cell tumor, while its juxtaposition to *ATF1* and *CHOP* leads to angiomatoid fibrous histiocytoma and myxoid liposarcoma, respectively. *EWS* can replace *TLS/FUS* in the *FUS/CHOP* chimera detected in myxoid liposarcoma and *TAF2N* in the *TAF2N/TEC* chimera in extraskeletal myxoid chondrosarcoma.

*EWS*-associated tumors. Detection of these chimeric transcripts can be performed by conventional cytogenetics, by molecular cytogenetics (FISH with painting probes or gene-specific probes), and at the transcriptional level by RT-PCR, and it has formed the basis of a sensitive and specific diagnostic assay for these tumors. Schematic representations of the most important translocations are provided in Figs. 6 and 7.

### Soft Tissues

#### *EWS-FLI1 t(11;22)(q24;q12)*

Karyotypic analyses have revealed a tumor-specific chromosomal translocation *t(11;22)(q24;q12)* in 86% of both Ewing's sarcoma (ES) and pPNET. The (11;22) translocation results in the fusion of the N-terminal region of the *EWS* gene rich in

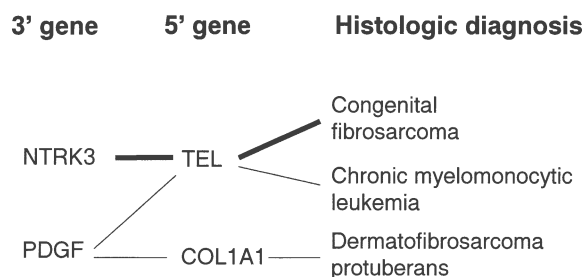


Fig. 7. *PDGF* and *NTRK3* rearrangements. *PDGF* may have more than one partner gene leading to different histologic entities. Interestingly, the same chimera, *PDGF-TEL* is carried by congenital fibrosarcoma and chronic myelomonocytic leukemia.

Glu, Ser, and Tyr residues to the *ETS*-like DBD of the *FLI1* gene. *EWS* is an ubiquitously expressed gene located on chromosome 22 that encodes for an RNA-binding protein, whereas *FLI1*, located on chromosome 11, is a member of the *ETS* family of transcription factors. The oncogenic effect of the t(11;22) translocation is caused by the formation of a chimeric protein. The protein has the potential to promote tumorigenesis by acting as an aberrant transcription factor that is functionally distinct from the normal *FLI1*. The COOH-terminal domain in addition to the *FLI1* DBD is necessary to promote cellular transformation (388). A potential regulatory site within the *EWS* IQ domain at Ser266 has been identified, and it was demonstrated that phosphorylation of this serine regulates *EWS* transcriptional activity (389).

Several different *EWS-FLI1* fusion types (up to 18 possible types) have been observed; two types, fusion of *EWS* exon 7 to *FLI1* exon 6 (type 1) and fusion of *EWS* exon 7 to *FLI1* exon 5 (type 2), account for approx 85% of *EWS-FLI1* fusions (390). Type 1 *EWS-FLI1* fusion has been shown to be a significant positive predictor of overall survival in patients with localized disease (390). This trend was confirmed by a subsequent study in which it was demonstrated that differences in the C-terminal partner of *EWS* fusions are not associated with significant phenotypic differences (391). Thus, molecular detection of the t(11;22) translocation and the molecular characterization of the fusion types are valuable in the differential diagnosis of small round cell tumors and provide information for the staging and prognosis of ES. *EWS-FLI1*-positive cells were amplified by RT-PCR in bone marrow and peripheral blood of a subset of patients with both nonmetastatic and metastatic ES or PNET, a finding that suggests a possible application of RT-PCR to the early identification of patients who may benefit from alternative therapy or who may be spared overtreatment (392). Nonrandom chromosomal aberrations were found associated with this translocation in more than 50% of these tumors. Chromosome gain is the most frequent event, of which trisomy 8 is the most common observed. Gain of chromosomes 2, 12, and 20 was reported with the unbalanced translocation t(1;16) and deletion of the short arm of chromosome 1. Studies analyzing lifetime survival demonstrated that a complex karyotype with multiple chromosomal aberrations is associated with poorer outcome in ES (393).

#### *EWS-ERG* t(21;22)(q22;q12)

t(21;22) is a variant translocation of *EWS* gene present in 5% of patients with ES. This translocation gives origin to the fusion of *EWS* to a member of the *ETS* family on chromosome 21. In this translocation, identical *EWS* nucleotide sequences found in the *EWS-*

FLI1 fusion transcripts are fused to portions of ERG encoding an ETS DBD resulting in the expression of a hybrid EWS-ERG protein (394). Recent findings suggest that EWS-ERG fusions in these sarcomas may be generated by an inversion of the *ERG* gene or a part thereof followed by an insertion into the *EWS* gene on the der(22) (395).

#### EWS-ETV1 *t*(7;22)(p22;q12)

*t*(7;22)(p22;q12), a rare variant chromosomal translocation, identified in two pPNET cases (396,397), fuses *EWS* to the *ETV1* gene, a member of the ETS family of transcription factors located on chromosome 7p22. Identical *EWS* nucleotide sequences found in most *EWS-FLI1* and *EWS-ERG* chimeric transcripts are fused to a region of *ETV1* encoding an ETS domain with sequence-specific DNA-binding activity.

#### EWS-FEV *t*(2;22)(q33;q12)

*EWS* can be fused to *FEV* in the chromosomal translocation *t*(2;22) in a subset of ES. The *FEV* gene is located on chromosome 2 and consists of three exons. In the chimeric transcript, exon 10 of *EWS* fuses within intron 1 of *FEV*. *FEV* is an additional member of the ETS family that encodes a 238 amino acid protein containing an ETS DBD closely related to that of *FLI-1* and *ERG*. Compared with *FLI-1* and *ERG*, *FEV* lacks transcription regulatory domains in its N-terminal part. The C-terminal part of *FEV* is Ala rich, suggesting a potential transcription repressor activity. *FEV* expression is detected in adult prostate and small intestine, but not in other adult or fetal tissues (398). A new fusion transcript type, in which exon 7 of *EWS* gene is fused with exon 2 of *FEV*, has recently been reported, supporting the existence of a heterogeneity of molecular rearrangements (23).

#### EWS-E1AF *t*(17;22)(q12;q12)

The *t*(17;22) chromosomal translocation, leading to the fusion of *EWS* with *E1AF*, was described in an undifferentiated sarcoma of infancy. *E1AF* is a newly isolated member of the ETS family of genes that is located on chromosome 17q21 and encodes for the adenovirus E1A enhancer-binding protein. The breakpoint on chromosome 17 lies in the region upstream of the ETS domain of the *E1AF* gene.

The human *E1AF* gene is organized in 13 exons distributed along 19 kb of genomic DNA. Its two functional domains, the acidic domain and the DNA-binding ETS domain, are each encoded by three exons. The 3'-untranslated region of *E1AF* is 0.7 kb. The 5'-untranslated region is approx 0.3 kb and is composed of a first exon upstream of the exon containing the first Met (399). As in other fusion proteins previously characterized in the Ewing family sarcoma, it is assumed that the RBD of *EWS* may be replaced by the DBD of *E1AF*.

#### EWS-ZGS *t*(1;22)(p36.1;q12)

A new translocation was detected in a CD99-positive, MIC2-negative multidirectional differentiated small round cell sarcoma involving the *EWS* gene and a new gene located at 22q12. This new gene, named *ZSG* (zinc-finger sarcoma gene), is a putative Cys2-His2 zinc-finger protein that contains a POZ transcriptional repressor-like domain at the N-terminus. The translocation rearranges intron 8 of *EWS* and exon 1 of *ZSG*, generating a fusion sequence that comprises the transactivation domain of *EWS* fused to the zinc-finger domain of *ZSG*. This product lacks the transcriptional repressor domain at the N-terminus of *ZSG*. This rearrangement, undetectable by cytogenetics, activates *EWS* in soft-tissue sarcoma (400).

**EWS-ATF1 *t*(12;22)(q13;q12)**

The translocation *t*(12;22)(q13;q12) is frequently and specifically found in malignant melanoma of soft tissues also named clear cell sarcoma and causes the fusion of *EWS* to the transcription factor *ATF1*. The chimeric EWS-ATF1 protein consists of the N-terminal domain of EWS linked to the  $\beta$ ZIP DBD of *ATF1* (401).

The resulting fusion protein that causes malignant melanoma of soft parts, by *trans*-cooperating with small regions of the EWS activation domain (EAD), (approx 30 residues), results in a potent transcriptional activation dependent on the conserved tyrosine residues present in degenerate hexapeptide repeats (DHRs, consensus SYGQQS). These findings provide evidence for a role of DHRs in EAD-mediated *trans*-activation and demonstrate that the EAD represents a novel tyrosine-dependent transcriptional activation domain (402). Moreover, it was demonstrated, both *in vitro* and *in vivo*, that EWS-ATF1 associates constitutively with CBP, a transcriptional coactivator, which links various transcriptional factors to basal transcription apparatus and participates in transcriptional activation, growth, cell-cycle control, and differentiation (403).

**EWS-WT1 *t*(11;22)(p13;q12)**

The translocation *t*(11;22)(p13;q12), recurrently associated with desmoplastic small round cell sarcoma, juxtaposes *EWS* to the Wilms' tumor gene *WT1* on chromosome 11p13. *WT1* encodes a zinc-finger transcription factor that may have a crucial role in normal genitourinary development. It is expressed in the developing kidney, gonads, spleen, mesothelium, and brain. *WT1* is an oncosuppressor gene specifically inactivated in a subset of Wilms' tumors, and mutations have been found in the germline of susceptible individuals. In the *EWS-WT1* rearrangement, the breakpoints involve the intron between *EWS* exons 7 and 8 and the intron between *WT1* exons 7 and 8, producing an in-frame fusion of the functional domains of the two genes, even if a molecular heterogeneity of *EWS-WT1* fusion transcripts has been described (404). The chimeric protein consists of the N-terminal domain of *EWS* and the DNA-binding zinc-finger domain of *WT1*. The EWS-WT1 chimera may encode transcriptional activator target genes that overlap with those repressed by WT1 (405). Expression of the fusion transcript induces the expression of endogenous DGFA (406) and of interleukin-2 (IL-2) and IL-15 (407).

**EWS-CHN *t*(9;22)(q22-31;q11-12), RPB56-CHN *t*(9;17)(q22;q11.2)**

A recurrent translocation, *t*(9;22)(q22;q12), has been recognized in extraskeletal myxoid chondrosarcoma (EMC). In this specific translocation, the *EWS* gene becomes fused to *CHN*, a novel orphan nuclear receptor with a zinc-finger DBD located at 9q22-31. *CHN* (also referred to as *TEC*, *NOR-1* or *MINOR*) appears to be the human homolog of the rat gene *NOR1*, which was identified as a sequence overexpressed in brain cells undergoing apoptosis. The chimeric *EWS-CHN* gene encodes an *EWS-CHN* fusion protein in which the C-terminal RBD of *EWS* is replaced by the entire *CHN* protein, including a large N-terminal domain, a central DBD, and a C-terminal ligand-binding/dimerization domain. EWS-CHN fusion protein induces tumorigenesis in EMC by activating the expression of *CHN* target genes, but no putative target genes have been identified so far (408).

To date, three variants of EWS-CHN fusion transcript have been identified: type I, type II, and type III transcripts (409). In an RT-PCR assay, using paraffin-embedded specimens, *EWS-CHN* or *RBP56-CHN* fusion gene transcripts could be detected in 15 (83%) of the 18 cases: *EWS-CHN* type 1 in 11 cases, *EWS-CHN* type 2 in 1, and



*RBP56-CHN* in 3 (410). The *EWS/CHN* type I transcript appears to be the most represented in EMC.

Two cases of EMC expressed a novel translocation  $t(9;17)(q22;q11.2)$  in which exon 6 of *RBP56* gene (referred to also as *TAF2N* or *hTAFII 68*) was fused to the entire coding region of *CHN (TEC)* (411). This transcript is structurally and functionally very similar to the *EWS-CHN* fusion. A case of EMC carrying histochemical, immunohistochemical, and ultrastructural evidence of neuroendocrine differentiation with the same translocation  $t(9;17)(q22;q11.2)$  has been reported (412).

#### *EWS-CHOP* $t(12;22)(q13;q12)$

$t(12;22)$  has been described in myxoid/round cell liposarcomas (413). This chromosomal translocation leads to fusion between the N-terminal part of *EWS* and the *CHOP* gene, creating an *EWS-CHOP* chimeric gene. *CHOP* maps on chromosome 12q13 and was previously demonstrated to be consistently involved in rearrangements with the *FUS* gene in the  $t(12;16)$  in myxoid/round cell liposarcomas. At the molecular level, the breakpoints on *EWS* occurred within intron 7, close to an ALU sequence, and, similarly, the breaks on *CHOP* were observed to cluster in intron 1 near ALU sequences (414). A variant transcript, consisting of exons 1–10 of *EWS* and exons 2–4 of *CHOP*, was recently described (415).

The presence of the *EWS-CHOP* chimeric gene in myxoid/round cell liposarcomas indicates that the N-terminal part of *FUS* may be replaced by the N-terminal portion of *EWS* in a *Chop* fusion oncoprotein and that the two N-terminal parts, when fused to certain transcription factors, have a common or very similar oncogenic potential.

When comparing the clinicopathologic features of the  $t(12;22)$ -carrying myxoid/round cell liposarcoma cases with cases harboring the more usual  $t(12;16)$ , no clinical or pathologic differences were identified (413).

#### *FUS-CHOP* $t(12;16)(q13;p11)$

$t(12;16)(q13;p11)$  is characteristic of the human myxoid/round cell liposarcomas (413). This chromosomal abnormality results from the fusion between a gene on chromosome 16 called *FUS* or *TLS* and a gene on chromosome 12 that encodes for a dominant inhibitor of transcription, *CHOP*. The *FUS* product contains a Glu/Ser/Tyr-rich segment and an RBD, as in the *EWS* protein. After the rearrangement, the putative RBD of *FUS* is replaced by the entire *CHOP* coding region, which contains a basic Leu zipper domain. As in the *EWS* fusion, the *FUS* domain provides a transcriptional activation domain to a presumptive DNA-binding activity of *CHOP*. To date, seven chimeric transcripts have been reported, among which the transcript types 1 and 2 are the most common variants (416). In contrast to some other translocation-associated sarcomas, the molecular variability of *FUS-CHOP* fusion transcript structure does not appear to have a significant impact on clinical outcome of myxoid liposarcomas (417).

#### *FUS-ATF1* $t(12;16)$

One case of angiomatoid fibrous histiocytoma (AFH) was reported (418) carrying a translocation  $t(12;16)$  involving *FUS* gene on chromosome 16 and *ATF1* on chromosome 12. The fusion transcript, detected by RT-PCR experiments and subsequent direct sequencing, revealed that *FUS* gene was interrupted at codon 175 and fused to codon 110 of *ATF1*, resulting in an in-frame junction with a Gly to a Val (GGT to GTT) transition. Very recently, an identical fusion of *FUS* and *ATF1* has been detected in a large,

deep-seated AFH, suggesting that the resulting chimera may be characteristic of these tumors (419).

#### PAX3-FKHR *t(2;13)(q35;q14)* and PAX7-FKHR *t(1;13)(p36;q14)*

Alveolar rhabdomyosarcoma often harbors specific translocations, resulting in the fusion of a forkhead-domain gene *FKHR* at 13p14 with either the *PAX3* or *PAX7* developmental control genes at 2p35 and 13q14, respectively (420). *PAX3* and *PAX7* each encode a transcription factor with a DBD (paired box and homeodomain) that controls development by activating specific target genes. After translocation, the resulting chimeric transcription factor contains the DBD, a truncated *FKHR* DBD, and the C-terminal region of *FKHR*. A study of 171 childhood rhabdomyosarcoma patients (93 cases of embryonal and 78 of alveolar rhabdomyosarcomas) confirmed that these fusion transcripts are specific only for the alveolar histotype and showed that fusion status is not associated with outcome differences in patients with locoregional disease. By contrast, in patients with metastatic disease, the expression of *PAX3-FKHR* and *PAX7-FKHR* identifies a very high-risk subgroup and a favorable outcome subgroup, respectively (421).

#### SYT-SSX *t(X;18)(p11.2;q11.2)*

A characteristic *SYT-SSX* fusion gene resulting from the chromosomal translocation *t(X;18)(p11;q11)* is detectable in almost all (>90%) synovial sarcomas. As a result of this translocation, the *SYT* gene from chromosome 18 fuses to one of the three highly homologous genes *SSX1*, *SSX2*, or, rarely, *SSX4* at Xp11.2. Several variants of this translocation have been observed and among the very rare *SSX4* fusion type, two *SYTSSX4* fusion transcripts are reported with a different breakpoint in the *SSX4* gene (422). The formation of the corresponding chimeric genes, *SYT-SSX1*, *SYT-SSX2*, and *SYT-SSX4*, in which the C-terminal amino acids of *SYT* are replaced by amino acids from the C-terminus of the *SSX* proteins, leads to the expression of fusion proteins whose function is still unclear, but likely they act as aberrant transcriptional regulators (423). A co-existence of *SYT-SSX1* and *SYT-SSX2* fusion transcripts has very recently been observed in 10% of the 121 synovial sarcomas investigated (424), revealing a new heterogeneous feature of this tumor type.

Increasing evidence has implicated that *SYT-SSX* could play an important role in development and progression of synovial sarcoma (423). A recent multicenter study reported that overall survival was significantly better among cases localized at diagnosis, carrying the *SYT-SSX2* transcript, and among patients with primary tumors <5 cm in greatest dimension. The impact of fusion type on survival remained significant when stratified for primary tumor size but was no longer significant when stratified for disease status at presentation (425). Notably, it was reported that *SYT-SSX1* fusion transcript appeared to be an independent, prognostic factor clearly associated with a reduced metastasis-free survival (426).

#### PDGFB-COL1A1 *t(17;22)(q22;q13)*

The chromosomal translocation *t(17;22)(q22;q13)* was identified in dermatofibrosarcoma protuberans, an infiltrative skin tumor of intermediate malignancy. This tumor, and its juvenile form, giant cell fibroblastoma, are cytogenetically characterized by the presence of supernumerary ring(s) derived from the *t(17;22)* (427). The breakpoints from translocations and rings contain the fusion of PDGFβ chain and the *COL1A1* gene (428).

*PDGFB* (*c-sis* protooncogene) has transforming activity and is a potent mitogen for several cell types. *COL1A1* is a major constituent of the connective-tissue matrix. The gene fusion, deleting exon 1 of *PDGFB*, leads to a deregulated production of PDGFB generating a stimulation of PDGFR and to malignant transformation. DNA transfection onto NIH3T3 fibroblast cells provided direct evidence of the transforming activity of *COL1A1/PDGFB* chimeric sequence (429). STI571, an inhibitor of PDGFR and ABL kinase activity, has been used in vitro to test its effects on cell growth. It was shown that the growth rate was reduced and the associated transformed phenotype changed to a flattened one. Interestingly, the effect could be reversed on removal of the compound (430). Three patients with metastatic disease were treated with STI571 (imatinab mesylate) (431,432). Two responded to the treatment, thus leading to a surgical resection of the tumoral mass. One did not respond to STI571 and rapidly died of disease. These clinical data indicate that STI may be useful for patients with local advanced disease, when other options for local therapy are limited (Fig. 7).

#### ETV6-NTRK3 *t*(12;15)(*p*13;*q*26)

Congenital fibrosarcoma is an uncommon soft-tissue tumor mainly involving the extremities of neonates, and cellular mesoblastic nephroma is a rare renal tumor generally diagnosed within the first 3 mo of life. Despite the diverse tissue origin, but in keeping with a very similar histologic appearance, the diseases share two specific cytogenetic abnormalities: trisomy of chromosome 11 and a *t*(12;15) translocation. This rearrangement creates a transcriptionally active fusion gene that encodes a chimeric oncoprotein, ETV6-NTRK3 (EN). This protein contains the helix-loop-helix dimerization domain of ETV6 (also referred to as TEL) fused to the tyrosine kinase domain of NTRK3. EN transforms NIH3T3 fibroblasts through a constitutive activation of both MAPK pathway and PI3K-AKT pathway (433) (Fig. 7).

#### TPM3-ALK *t*(2;5)(*p*23;*q*35)

Inflammatory myofibroblastic tumor is a rare mesenchymal neoplasm composed of fascicles of bland myofibroblasts admixed with a prominent inflammatory component. In this tumor, *ALK* is fused with the nontropomyosin gene (*TPM3*). The C-terminal tyrosine kinase domain of *ALK* is fused with the N-terminal coiled-coil domain of *TPM3*. The same translocation is shared by ALCL. As in ALCL, other partners of *ALK* have been identified, such as clathrin heavy-chain *CLTC* gene, localized to 17q23 and detected in only one case (434); *TPM4*; and *CARS*, which encodes the cysteinyl-tRNA synthetase (435) (Fig. 5).

### Epithelial Tissue

Cytogenetic and molecular analyses of thyroid tumors have indicated these neoplasms as a good model for analyzing human epithelial cell multistep carcinogenesis (436). The thyroid gland manifests a wide spectrum of malignant neoplasms, including medullary thyroid cancer, which develops from the neural crest-derived C cells, and tumors arising from the epithelial follicular cells. The latter comprise several tumor types with different phenotypic characteristics and variable biologic and clinical behavior.

Molecular studies have identified specific genetic alterations in these different tumor types. In particular, the well-differentiated carcinomas of the papillary type are characterized by activation of the *RTK RET* and *NTRK1* protooncogenes. Somatic rearrangements of both *ret* and *NTRK1* produce several forms of oncogenes (437), whereas the

well-differentiated carcinomas of the follicular type are characterized by the rearrangement PAX8-PPAR $\gamma$ . In all cases, *RET* or *NTRK1* tyrosine kinase domains are fused to the N-terminus of different genes. Detection methods include Southern blot, extra-long PCR, and RT-PCR.

### RET PTC1

The *RET-PTC1* oncogene, a chimeric transforming sequence, originates by chromosome 10 inversion, inv(10)(q11.2q21.2), and is generated by the fusion of the tyrosine kinase domain of *RET* to the 5'-terminal region of the gene *H4-D10S170*. *H4-D10S170* contains a coiled-coil sequence that confers to the oncoprotein the ability to form dimers, resulting in a constitutive activation of the tyrosine kinase function. A novel rearrangement has been described, which contains the N-terminal 150 residues of *H4* and creates an oncoprotein named *RET/PTC1L* with lower transforming activity than *RET/PTC1* (438).

### RET-PTC2

In the case of the *RET-PTC2* oncogene, the rearrangement involves the tyrosine kinase domain of *RET* and the gene of the regulatory subunit RI $\alpha$  of PKA, which maps to chromosome 17q23 (439,440). Cytogenetic analysis has revealed that this oncogene arises from a t(10;17)(q11.2;q23) reciprocal translocation (427). RI $\alpha$ , like the *H4* gene, contains a dimerization domain involved in the activity of the oncogene.

### RET-PTC3/PTC4

The *RET-PTC3* and *RET-PTC4* oncogenes are generated by the fusion of the tyrosine kinase domain of *RET* and a gene named *ELE1 $\alpha$ -ARA70* (also known as *RFG*) (441,442) located in the same region, 10q11.2. In this case, a paracentric inversion of the long arm of chromosome 10 occurs, with breakpoints in exon 5 of *ELE1 $\alpha$ -ARA70* and exon 12 (*RET-PTC3*) and 11 (*RET-PTC4*) of *RET*. Along with *RET-H4*, intrachromosomal rearrangements involving *RET* and *ELE1 $\alpha$ -ARA70* are very frequent events (58%) in thyroid cancers of children of the Chernobyl-contaminated zone. Moreover, an association exists between *RET-PTC3* rearrangement and short latency of PTC, particularly the solid-follicular variant.

### RET-PTC5-PTC9 and RET-PCM1 ELKS-RET

After the Chernobyl accident, an unusual higher frequency of thyroid cancers was observed in Belarus and Ukraine, and new forms of *RET* rearrangements were identified in cases of papillary cancer from contaminated areas. In these new oncogenes, *RET* was fused to seven donor genes. For instance, *RET-PTC5* fusion partner protein is GOLGA5, a coiled-coil protein expressed on the Golgi surface (443). *RET-PTC6* and *RET-PTC7* display rearrangements with the transcriptional intermediary factor 1- $\alpha$  and  $\gamma$ , respectively (444). This protein family is able to bind the ligand-dependent activation function (AF2)-activating domain of the estrogen receptor, RARs, RXRs, and vitamin D3 receptor, and to regulate transcription. The *RET* fusion partner in *RET-PTC8* is kinectin (445), whereas in *RET-PTC9*, *RET* rearranges with REG9, a putative cytoplasmic protein possibly involved in intracellular-transport processes (446). In *RET-PCM-1*, the activating sequences belong to a gene coding for a centrosomal protein that displays distinct cell-cycle distribution (447). The *ELKS* gene (rearranged in the *ELKS-RET* oncogene) is transcribed into an ubiquitously expressed mRNA with unknown function (448).

## NTRK1

*NTRK1*, located on chromosome 1q22 (449), encodes one of the receptors for nerve growth factor. *NTRK1*, originally detected in a human colon carcinoma as an oncogene, was generated from the chromosomal rearrangement by fusing the *NTRK1* tyrosine kinase domain to sequences of a tropomyosin gene, *TPM3*.

The extracellular region of NTRK1 protein contains three Leu-rich motifs flanked by conserved Cys residues. This extracellular domain contains two C2 Ig-like loops similar to those present in NCAMs and in receptors for fibroblast growth factors, PDGF, and CSF-1. NTRK1 is expressed primarily in the nervous system and appears essential for the development of both the peripheral and central nervous systems (436).

## TRK

The *TRK* oncogene is generated by a 1q intrachromosomal rearrangement involving an isoform of the nonmuscle tropomyosin (*TPM3*) mapped to chromosome 1q31 and NTRK1 (450). Molecular analysis revealed the presence not only of the product of the oncogenic rearrangement (5' TPM3-3' NTRK1), but also of that related to the reciprocal event (5' NTRK1-3' TPM3).

## TRK-T1

The *TRK-T1* (T2;T4) oncogene, formed by the fusion of *NTRK1* tyrosine kinase domain to sequences of the translocated promoter region (TPR) gene localized on chromosome 1q25 (451), generates 3e chimeric transforming sequences. TRK-T1 is encoded by a hybrid mRNA that contains 598 nucleotides of *TRP* and 1148 nucleotides of *NTRK1*. An inversion of 1q is responsible for formation of TRK. *TRK-T2* and *TRK-T4* rearrangements involve different genomic regions of the two partner genes, *TPR* and *NTRK1*, but occur in the same intron of both these genes. As a consequence, the same mRNA and oncoprotein are produced in both cases. The molecular characterization of these rearrangements indicates that the chromosomal mechanism leads to oncogenic activation as an inv(1q) (452).

## TRK-T3

The *TRK-T3* oncogene contains 1412 nucleotides of *NTRK1* preceded by 598 nucleotides belonging to a novel gene, *TGF* (TRK-fused gene), located on chromosome 3. The latter gene displays a coiled-coil region that could confer to the oncoprotein the ability to form complexes (436).

The most significant clinical relevance of *RET* rearrangements is the correlation with radiation exposure. In fact, a significant proportion of papillary thyroid carcinomas from children exposed to the consequences of the Chernobyl nuclear accident contain a rearranged form of *RET* (453,454).

In addition, a correlation between the combination of *RET* and *NTRK1* positivity and young age of patients at diagnosis and a significant association between *RET*-*NTRK1* positivity and locally advanced stage of disease at onset (pT4:  $p < 0.015$ ) has been reported (455). A multivariate analysis confirmed that *RET*-*NTRK1* activation parallels an unfavorable disease presentation, which may correlate with less favorable disease outcome, and also showed that within these tumors, the frequency of *RET*-*NTRK1* positivity occurs irrespective of subtypes and degree of differentiation (456).

### PAX8-PPAR $\gamma$ t(2;3)(q13;p25)

A translocation t(2;3)(q13;p25), detected in a subset of human thyroid follicular carcinomas, results in fusion of the DBDs of the thyroid transcription factor PAX8 to domains A–F of the PPAR $\gamma$ 1. Since *PAX8* gene can undergo to splicing events, different transcripts can be originated after the fusion with *PPAR $\gamma$*  gene (457). PAX8-PPAR $\gamma$ 1 mRNA and protein were first detected in 5 of 8 thyroid follicular carcinomas but not in 20 follicular adenomas, 10 papillary carcinomas, and 10 multinodular hyperplasias (458). Interestingly, a subsequent investigation performed on 118 thyroid tumors confirmed that PAX8-PPAR $\gamma$  rearrangement segregated with follicular neoplasms, as it was not found in others types of malignant or benign thyroid lesions, including papillary carcinoma and its follicular variant, Hürthle cell carcinoma, and Hürthle cell adenoma, without follicular malignancies (457,459).

Since the resulting protein PAX8-PPAR $\gamma$ 1 inhibits the thiazolidinedione-induced transactivation by PPAR $\gamma$ 1 in a dominant-negative manner, PAX8-PPAR $\gamma$ 1 may be useful not only in the diagnosis but also in the treatment of thyroid carcinoma (458).

### New Translocations Not Fully Characterized

In a pediatric renal tumor characterized by features of both carcinoma and epithelial angiomyolipoma, the translocation t(6;11)(p21.1;q12) has been described. To date, no molecular characterization of the genes involved in the fusion is available (460).

t(15;19)(q13;p13.1) identifies a particularly aggressive form of a carcinoma in children and young adults (461). This carcinoma is poorly differentiated histologically and carries an unfavorable prognosis. The partial molecular characterization has revealed that *BDR4* (bromodomain-encoding) gene on chromosome 19 is interrupted. The breakpoint on chromosome 15 has been localized, identifying several candidate oncogenes that might represent the *BDR4* fusion partner.

## Conclusion

During the last few years, a gene-by-gene comparative analysis of normal and tumor tissues allowed researchers to acquire new insights into tumor pathogenetic pathways and biologic functions of molecular markers. These acquisitions resulted in less empiric and more biologically oriented approaches to tumor classifications as well as to clinical use of biomarkers. Classifications based on genetic profile successfully complemented the morphologic ones, and several biomarkers introduced in the clinical setting to improve diagnosis and prognosis became or are becoming targets of new, more tailored treatments. The interest has shifted from the molecular markers to their molecular pathways that can be targets for direct or indirect therapeutic interventions. Since more cancers are treated at every stage of the disease and individual mutations may alter the response to therapy, the concept of a prognostic factor that is independent of therapy is disappearing. As a consequence, a number of molecular markers or, better, the associated pathways of these molecules are regarded as targets for therapy rather than diagnostic/prognostic predictors.

A number of chromosomal translocations correctly categorize several sarcomas and lymphomas lacking morphologic unequivocal features. This is true for the differential diagnosis of PNET and alveolar rhabdomyosarcoma among the so-called small round cell sarcomas, and for synovial sarcoma among the spindle cell sarcomas. Regarding hematopoietic tumors, the assessment of the specific translocations may assist the diagnostic definition of mantle cell lymphoma, LCAL, and APL. From the prognostic point

of view, the presence of the type 1 of *EWS-FLI1* and *EWS-ERG* or *SYT-SSX2* transcripts seems to predict a more favorable clinical outcome in PNET and synovial sarcomas, respectively.

Mounting evidence points out the possible application of the assessment of *TP53* and *K-RAS* mutations in sputum and *TP53*, *K-RAS*, and *APC* mutations in stool specimens for a noninvasive presymptomatic diagnosis of lung and colorectal carcinoma, respectively. These approaches, although promising, are limited by a low sensitivity and the use of expensive and time-consuming technologies.

Regarding the evolution of biomarkers from diagnostic/prognostic tools to molecular targets for therapeutics, the more relevant examples are represented by *Her-2/neu*, inhibitors of abnormally activated tyrosine kinases, ATRA, and COX2 inhibitors. In general, the concept of molecular targeting involves the interruption of interaction between cognate ligand-receptor or enzyme-substrate. *Her-2/neu* exemplifies the former situation. A number of MAbs were developed against this surface-located oncoprotein among which the humanized murine antibody trastuzumab has successfully been applied, alone or in association with chemotherapy, in the clinical setting. An example of the second type of inactivation is given by tyrosine kinase inhibitors such as STI571. This agent, recognized as a paradigm of new agents for cancer therapeutics, specifically inactivates the abnormal kinase activity of *BCR/ABL* oncogene in CML and c-kit-activating mutations in GIST, exemplifying that the paradigm may be translated to other malignancies sustained by different molecular alterations but sharing the same enzymatic abnormality. ATRA therapy is another example of therapy targeting a specific receptor system, the RARA receptor that retains its LBD in the chimeric PML/RARA oncoprotein form, a molecular hallmark of APL. By binding to the chimeric oncoprotein, ARA may restore the regulatory pathway, permitting the terminal differentiation of myeloid cells. A further example is represented by COX-2-selective inhibitors, such as celecoxib and refecoxib, which have been shown to inhibit apoptosis, angiogenesis, and cell proliferation, and to stimulate the immune system, and which are successfully applied in cancer treatment and prevention.

Given the prominence of apoptosis in determining therapeutic responses, it cannot be disregarded that most chemotherapy schemes currently applied are apoptosis dependent and that what in the past has been defined (drug resistance) is, at least in part, due to resistance to apoptosis. Thus, a more rational and effective application of current chemotherapy regimens cannot omit knowledge about the genetic makeup of a given tumor in terms of functional status of genes involved in the cell death pathway, such as *TP53* and *p16<sup>INK4</sup>*, and, to a lesser extent, *BCL2*.

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# 4

## Genetic Markers in Breast Tumors with Hereditary Predisposition

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### Introduction

Over the past two decades, we have come to an understanding of cancer as a genetic disorder caused by the progressive accumulation of multiple genetic changes, which include point mutations, chromosomal rearrangements, viral insertions, and genomic amplifications and deletions (1,2). Gene amplifications, point mutations, viral insertions, and chromosomal rearrangements are dominant genetic damages that primarily target oncogenes whose gain of function (overexpression) leads to dysregulation of cell growth and transformation. Recessive point mutations and deletions mainly cause loss of function in tumor suppressor genes (TSGs) that control cell-cycle progression and DNA repair mechanisms (2).

To date, with the exception of *RET* mutations in multiple endocrine neoplasia syndrome, inherited predispositions transmitted by heterozygous defects in TSGs are the sole form in which hereditary cancer syndromes have been identified. In germline mutation carriers, the loss of the remaining normal allele of a TSG (loss of heterozygosity [LOH]) is required for tumorigenesis. Oncogene activation occurs frequently as secondary somatic changes in tumor development and these changes are molecular markers often targeted for therapy. We focused this review on the genetic alterations that are characteristic of hereditary breast cancer, since these appear to be the best characterized.

Although epidemiologic evidence supports certain risk factors (e.g., age, residence in Western countries, obesity, nulliparity, early menarche, alcohol consumption, ionizing radiation, hormone replacement therapy), a family history of breast cancer remains the strongest risk factor for the disease. Inherited forms comprise approx 15–25% of all breast cancers and apparently have distinctive pathogenesis determined by the particular susceptibility gene involved (3,4). While the susceptibility genes in most breast cancers developing in familial clusters have yet to be identified, it is estimated that between 4% and 10% are caused by germline mutations in *BRCA1* and *BRCA2* genes, or by rare hereditary cancer syndromes caused by mutations in other TSGs (*p53*, *PTEN*, *ATM*) and some mismatch repair genes (*MSH2* and *MLH1*). Inherited breast cancer has sev-

eral distinctive clinical features: early age at onset, lower than in sporadic cases; higher prevalence of bilateral breast cancer; and presence of associated tumors (ovarian, colon, prostate, endometrial carcinomas, and sarcomas) in affected individuals (5).

For information about breast cancer susceptibility genes, mutations, functions, and interacting proteins, we refer the reader to recent comprehensive reviews (6–17). To date, *BRCA1* and *BRCA2* are the most relevant genes and we describe here the phenotypes of *BRCA1*- and *BRCA2*-associated breast cancers and the role of cooperative oncogenes, TSGs, and other potential markers involved in their tumor progression. The other known genes, which contribute to the less-common inherited breast cancer syndromes, are provided in Table 1. While our understanding of the molecular pathways involved in hereditary breast cancer progression is still in its juvenile stage, some progress has been made in the search for secondary somatic genetic changes involved in hereditary tumor development.

### Molecular Pathology of *BRCA1* and *BRCA2* in Breast Cancer

*BRCA1* and *BRCA2* are breast cancer susceptibility genes, the mutant form of which predisposes to both breast and ovarian cancers (18–20). *BRCA1* and *BRCA2* function as classic TSGs on 17q12–21 and 13q12–13, respectively, and loss of the wild-type allele is required for tumorigenesis in mutation carriers (21,22). *BRCA1* and *BRCA2* encode multifunctional proteins, which together with other proteins contribute to homologous recombination, DNA damage response, and transcriptional regulation (8). Current evidence suggests that *BRCA1/2* are in a class of caretaker genes, which function in maintaining genetic stability (23). It is estimated that approx 5% of all cases of breast carcinoma are inherited in a dominant autosomal fashion, and most are associated with germline mutations at *BRCA1/2* (24). Most disease-related alleles at *BRCA1/2* identified to date result in protein truncations (see Breast Cancer Information Core at <http://research.nhgri.nih.gov/bic/>). Mutations in *BRCA1/2* do not directly result in tumor formation but instead cause genetic instability, and trigger further alterations, including inactivation of other TSGs and/or activation of oncogenes, leading cells to malignant transformation (25).

Clinicopathologic and histologic characteristics of *BRCA1*- and *BRCA2*-associated tumors differ from each other and both differ from age-matched breast cancers unselected for family history. *BRCA1*-associated tumors display distinct aggressive pathologic features, including early age of onset, high tumor grade (26,27), estrogen receptor (ER) and progesterone receptor (PR) negativity, lack of expression of the estrogen-responsive gene *pS2*, and high proliferation rate (27–33). Histologically, the tumors are ductal-invasive carcinomas, often of the medullary subtype with features such as prominent pushing margins and lymphocytic infiltrate (29,34). *BRCA1* carriers, in addition to a high risk of breast and ovarian cancer, have increased risk of prostate, colon, liver, and bone cancers (7).

The *BRCA2* tumor phenotype is heterogeneous and not well defined. While overall *BRCA2*-associated tumors are higher grade, the expression of estrogen receptor/progesterone receptor (ER/PR), pleomorphism, mitotic count, and age distribution are not always different from those in sporadic breast cancers (27,28,35,36). Histologically, *BRCA2*-associated tumors show reduction in tubule formation, the presence of continuous pushing margins, and an extensive intraductal component (36–38). *BRCA2* carriers have a lower risk of ovarian cancer compared with *BRCA1* carriers, but melanoma and higher risk of prostatic, pancreatic, gallbladder, pharynx, and stomach can-



cers, and, dramatically, a higher risk of breast cancer in men (7). The oncogenes and other TSGs contributing to multistep carcinogenesis in *BRCA1/2*-deficient cells are listed in Table 2.

### **Genomic Instability**

*Genomic instability* is a broad term used to designate genetic changes which include aneuploidy, chromosomal instability and chromosomal aberrations, DNA amplifications, centrosome amplifications, and micronuclei formation. Genomic instability likely results from a failure of coordination of S-phase and/or mitotic checkpoints (39). Genomic instability is a prominent feature of hereditary breast cancer.

Aneuploidy is a marker of tumor progression and prognosis. Several studies using flow cytometry and cytogenetics evaluated the DNA content of *BRCA1/2*-related mammary tumor cells compared with tumor cells from sporadic cases (35,40–43). Breast cancers occurring in *BRCA1* mutation carriers were significantly more aneuploid. Analysis of 90 cases from 26 families with *BRCA1* mutations (40) revealed that *BRCA1*-associated cancers had a significantly lower diploidy rate than in the control group of 187 cases (13 vs 35%;  $p = 0.002$ ), lower mean aneuploid DNA index (1.53 vs 1.73;  $p = 0.001$ ), and higher aneuploid mean S-phase fraction (16.5 vs 9.3%;  $p < 0.0001$ ). The same conclusion was reported in patients with *BRCA1*-positive tumors which were more often DNA nondiploid (20/21 [95%]) compared with (18/34 [53%]) *BRCA1*-negative hereditary tumors, as well as being characterized by higher S-phase fraction values (42). The tumor cell line HCC1937, established from a primary breast carcinoma from a patient with a germline *BRCA1* mutation, was also found to be marked by multiple additional genetic changes including a high degree of aneuploidy, an acquired mutation of *TP53* with wild-type allele loss, an acquired homozygous deletion of the *PTEN* gene, and LOH at multiple loci known to be involved in the pathogenesis of breast cancer (44).

Using comparative genomic hybridization (CGH) accompanied by fluorescence *in situ* hybridization (FISH), cDNA microarrays, or flow cytometry, genome wide analyses of chromosomal regions that are either amplified or deleted in *BRCA1/2* breast cancers were done (41,45,46). One study (41) compared 21 *BRCA1*-related breast cancers and 15 *BRCA2*-related breast cancers with 55 unselected controls and showed that the total number of genetic changes was almost twice as high in *BRCA1* and *BRCA2* tumors. Ninety-two percent of *BRCA1*-associated and 62% of *BRCA2*-associated tumors were aneuploid vs 49% of control cancers. Meanwhile, the ploidy analysis of *BRCA1/2* tumors performed by three other research groups (35,40,43) gave conflicting results. Unlike *BRCA1* cases, the *BRCA2*-associated tumors were lower grade, were more often diploid with low aneuploid S-fraction, and were comparable to sporadic cases.

Analysis of chromosome instability (41) revealed that in *BRCA1*-associated tumors, losses of 2q, 4p, 4q, 5q, and 12q, and gains of 6p, 10p, and 17q22–24 were significantly more common than in the control group. Gains in the 17q22–24 segment were shared by both *BRCA1*- and *BRCA2*-associated tumors and were observed in 40 and 87% of cases, respectively, vs 8 to 31% in sporadic controls (41). In addition, both women and men with *BRCA2* were characterized by a higher frequency of 13q (73%), 6q (60%), 11q, and 9p losses, and gains of 20q13 (60%), 8q22–24, and 17q22-ter compared with the prevalence of these changes in the control group (12–18%) (45). Chromosomal aberrations which included both structural and numerical changes were highly prevalent at chromosomes 1, 3, 16, and 17 (43).

**Table 1**  
**Breast Cancer Susceptibility Genes**

Gene	Location	Protein function	Associated syndrome	Mode of inheritance	Mutation frequency	Risk range	Disease penetrance	Founder mutations	New mutations
<i>BRCA1</i>	17q12-21	DNA repair, transactivation	Hereditary breast/ovarian cancer Bilateral/multifocal breast tumor; risk of prostate colon, liver, and bone cancer	Autosomal dominant	approx 3% in White, <1% in African-Americans, 4-5% in populations with founder mutations; 12% of women by age 32; 20-40% of familial breast cancer	56-87% (by age 70); 25-60% risk of ovarian cancer	High	Common	Uncommon
<i>BRCA2</i>	13q12-13	DNA repair, transactivation	Hereditary breast/ovarian cancer Male breast cancer; risk of melanoma and pancreatic, gall-bladder, pharynx, stomach, and prostatic cancer	Autosomal dominant	3% in White, 1% in African-Americans, higher in populations with founder mutations; <2% of all breast cancer, 14% of all male breast cancer; 2.7% of women by age 32; 10-30% of familial breast cancer	37-84% (by age 70); 27% risk of ovarian cancer	High	Common	Uncommon
<i>TP53</i> <i>hCHK2/</i> <i>CHEK2</i>	17p13.1	Cell-cycle regulation	Li-Fraumeni syndrome Soft tissue sarcoma, breast cancer, central nervous system tumors, adrenocortical cancer, leukemia	Autosomal dominant	<1% of familial breast cancer	50-89% (by age 50), 100% (lifetime)	High Low	Less common	Common

<i>PTEN</i>	10q23	Phosphatase	Cowden syndrome 30–50% incidence of breast cancer; hamartoma; thyroid, oral mucosa, and brain tumors	Autosomal dominant	<1%	25–50% (by age 50)	Moderate	?	?
<i>MSH2</i> <i>MLH1</i>	2p22–21 3p21.3	DNA MMR	Muir-Torre syndrome Colorectal carcinoma	Autosomal dominant	<1% of familial breast cancer	12% (lifetime)	High	?	?
<i>STK11</i> / <i>LKB1</i>	19p13	Serine/reonine kinase	Peutz-Jeghers syndrome Hamartous polyps, breast, ovarian, and colon carcinoma	Autosomal dominant	<1%	High	High	?	?
<i>ATM</i>	11q22.3	DNA repair	Ataxia telangiectasia Leukemia, lymphomas, immuno- deficiency, ovarian cancer, 3.3–3.9% risk of breast cancer	Autosomal recessive	1%	Moderate	Low	Probably common	Probably uncommon

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References: 7, 11, 38, 151, 162–166

**Table 2**  
**Specific Somatic Genetic Alterations in Hereditary BRCA1/2-Associated Breast Cancers**

Gene/region	Modification	Frequency of Alteration		References
		BRCA1-associated breast cancer	BRCA2-associated breast cancer	
Tumor suppressor genes				
• <i>TP53</i>	Mutation/inactivation	42–68%/44–77% (vs 19–35%/22–35% sporadic)	Data conflicting: either 29–64%/45% (vs 17–19%/35% sporadic) or similar to sporadic	30, 31, 33, 34, 36, 42, 43, 61, 63, 64, 66, 67
• <i>E-cadherin</i>	Mutation/inactivation	Low, similar to sporadic	Low, similar to sporadic	31, 81, 82
Oncogenes				
• <i>C-MYB</i>	Amplification/overexpression	29% (vs 2% sporadic)	Low, similar to sporadic	83
• <i>TBX2</i>	Amplification/overexpression	46–62% (vs 8% sporadic)	15–85%	97
• <i>C-MYC</i>	Amplification	61% (vs 27% sporadic)	Not reported	104, 105
• <i>HER-2/neu</i>	Amplification/overexpression	0%/0–3% (vs 15%/15–19% sporadic)	Data conflicting: either NR/3% or similar to sporadic	31, 33, 36, 61, 63, 67, 118
• <i>K-RAS</i>	Mutation/overexpression	Similar to sporadic	Similar to sporadic	61
Cell cycle and apoptotic proteins				
• P16	Mutation/inactivation	Similar to sporadic	Similar to sporadic	61, 125–137
• P21	Deregulation	Data conflicting: overexpression either in 35% (vs 10% sporadic) or similar to sporadic	Data conflicting: overexpression either in 23% or similar to sporadic	31, 61
• P27	Inactivation	Data conflicting, presumably high	Data conflicting, presumably high	32, 66, 163
• Cyclin D1	Amplification/overexpression	Low, 0–11% (vs 26–63% sporadic)	High, 27–70%	28, 31, 82, 106, 141
• Cyclin E	Overexpression	Similar to sporadic	Not reported	82
• Bcl-2	Overexpression	Data conflicting: either low (30 vs 90% control) or similar to sporadic	Data conflicting: either high or similar to sporadic	31, 66, 139

Cytoskeletal proteins and proteases				Not reported	149
	•Cytokeratins 5/17	Expression	High	High	106, 149
	•Cytokeratin 8	Expression	Low	Similar to sporadic	31, 66
	•Cathepsin D	Overexpression	Similar to sporadic		
Steroid hormones and genes involved in endocrine signaling pathways					
	•ER/PR	Expression	Low	High, often similar to sporadic	27, 28, 31, 35, 36, 40, 42, 63, 66, 67, 164–166
•PS2	Expression	Low	High		28, 30, 21
Proliferative markers					
	•Ki-67	Expression	High, 83% (vs 48% sporadic)	Low or similar to sporadic	31, 82
•PCNA	Expression	High, similar to sporadic		High, similar to sporadic	31
Global genetic lesions					
	•Aneuploidy	Highly aneuploid	87–95% (vs 49–65% sporadic)	Data conflicting: either 62% or similar to sporadic	35, 40–43
•Chromosomal aberrations	Specific gains			8q22–24, 17q22–ter, 20q13	
•Centrosome aberrations	Amplification		Specific losses	6p, 10p, 17q22–24	41, 45–47, 49, 50
•Gene expression profiles	Downregulation/upregulation		3p, 3q, 5q, 12q	6q, 9p, 11q, 13q	25, 51–53
			25–30% (murine model)	44–65% (murine model)	
			Specific set of 9–176 genes	Specific set of 11–176 genes	106, 107
Epigenetic lesions					
	•Gene methylation profiles	Hypermethylation of gene promoter CpG islands	Low in <i>GSTP1</i> gene	High in <i>p16</i> gene	127
•Global genome demethylation	M5dC DNA content		42%, similar to sporadic	30%, similar to sporadic	127

Lodewyk et al. (46) used CGH on 28 *BRCA1* germline mutation carriers and 42 breast tumors from patients with family history with unknown *BRCA* status. They provided evidence that *BRCA1*-associated breast carcinomas exhibit specific somatic genetic aberrations. Based on somatic genetic CGH profiles, they developed a molecular classifier to distinguish *BRCA1* mutation carriers from non-*BRCA1* carriers with accuracy of 84%. The chromosomal bands used by this classifier include regions on chromosomes 3p, 3q, and 5q. Lodewyk et al. (46) also confirmed the loss of 5q and 12q found by Tirkkonen et al. (41); however, losses of chromosomes 4q and 2p were not confirmed. One of the explanations for the discrepancies is that the goal of the study by Lodewyk et al. (46) was to go beyond the identification of specific aberrations and to develop a classifier that would allow identification of individual *BRCA1* tumors within a group of high-risk patients. Preliminary data show that *BRCA2* tumors displayed specific genetic similarities with *BRCA1* tumors for some regions used by the classifier. This finding is not completely surprising because *BRCA1* and *BRCA2* are interacting proteins involved in overlapping molecular pathways.

Based on CGH data by Tirkkonen et al. (41) showing that 4p, 4q, and 5q are frequent targets of losses in *BRCA1* breast cancers, Nathanson et al. (47) investigated the suggestion that the modifier genes of *BRCA1* penetrance may be located in regions of allelic imbalance in the tumors of *BRCA1* mutation carriers. Using nonparametric linkage analysis, they observed a significant linkage signal at D5S1471 on chromosome 5q ( $p = 0.009$ ) in all the families analyzed together, and the significance increased in a subset of families with an average age of breast cancer diagnosis of <45 yr ( $p = 0.003$ ). These results suggest the presence of one or more genes on chromosome 5q33–34 that modify breast cancer risk in *BRCA1* mutation carriers such that they increase the age-adjusted penetrance of *BRCA1* mutations. Of the 25 genes within the region, *cyclin G1* (*CCNG1*) is of particular interest, because it is a transcriptional target of p53, which is known to interact with *BRCA1*, it localizes to nuclear foci after DNA damage, and it is upregulated in breast involution (47,48). No significant linkage to chromosome 4p or 4q was observed. These data suggest that a genomewide search to identify modifiers using this approach in a larger number of families with *BRCA1* mutations is feasible. One group had previously published data on modifier genes on 9p23–24 sequences that may influence the risk of male breast cancer conferred by *BRCA2* (49,50).

A significant finding from murine models was that *BRCA1*- and *BRCA2*-mutant cells carry out centrosome amplification (51,52). Analyzing either *BRCA1* $\Delta 11/\Delta 11$  mouse embryo fibroblast (MEF) cells or *BRCA1* conditional mutant mammary tumor cells, Xu et al. (51) and Weaver et al. (52) found that approx 25–30% of *BRCA1*-deficient cells contained supernumerary functional centrosomes, instead of only one or two as observed in the control cells. In the mitotic phase, these centrosomes formed spindles with multiple poles. The multipolar spindles pulled chromosomes in different directions, leading to unequal segregation of chromosomes and micronuclei formation. Consequently, the mutant cells were aneuploid. Centrosome amplification in MEFs derived from mice *BRCA2* $\Delta 11/\Delta 11$  impaired in DNA double-strand break repair (DSBR) was observed in 44% of cells in passage 2 and 65% in passage 3 instead of only 10% in control cells (53). In addition to chromosome aberrations, mutant cells frequently develop micronuclei (32% in passage 2 and 52% in passage 3 vs 3–4% in controls). These abnormal DNA-containing bodies were formed through loss of acentric chromosome fragments and by chromosome misaggregation, which resulted in aneuploidy (53). Considering that *BRCA1* protein can physically associate with the centrosome

(54–56), and that BRCA1 and BRCA2 have both protein–protein interaction and transactivation activities (16,17), these studies provide genetic evidence implicating *BRCA1/2* in the centrosome duplication process, either directly or indirectly, through transactivation of centrosome-specific genes. Although analyses of centrosome amplification in human specimens of hereditary breast cancer remain to be conducted, functional studies of BRCA1/2 proteins (9,54,56) and murine models (25) suggest that BRCA1/2 may have a functional role at mitotic centrosomes through the regulation of centrosome duplication/integrity, mitotic spindle formation, and proper segregation of chromosomes during mitosis, and that it helps to maintain the fidelity of cell division and preserve genomic stability. In hereditary breast cancers, some aspects of centrosome amplification may have clinical diagnostic and/or prognostic value, and it may be a potential target for cancer therapy (57,58).

Observations of definitive increase in genomic instability in BRCA1/2-deficient cancer cells, including aneuploidy, specific chromosome gains and losses, chromatid breaks and aberrant chromatic exchanges, deficient G1-S and G2-M checkpoints, and centrosome amplification and aberrant mitoses, as well as the implication of BRCA1/2 in the DNA repair process, homologous recombination and cell cycle control, are consistent with the proposed role for *BRCA1* and *BRCA2* genes as caretakers of the genome (23).

### ***p53 and Other TSGs***

The *TP53* TSG is the most frequently altered gene in human malignancies. p53 is a transcription factor and it regulates cell proliferation and apoptosis (59). Immunohistochemical assays revealed that approx 15–40% of sporadic breast cancers showed detectable expression of p53, resulting from accumulation of mutations in the *p53* (33). A number of studies (using either immunohistochemical [IHC] assays or DNA sequencing) have demonstrated a higher frequency of *p53* mutations or immunopositivity for p53 in *BRCA1/2*-associated breast cancers compared with control tumors (30,31,60–65). Overall, 53% of *BRCA1/2*-associated breast cancer cases overexpressed p53, compared with 24% of the sporadic cases ( $p < 0.0001$ ). *p53* mutations have been characterized in 42% of *BRCA1*-associated, 29% in *BRCA2*-associated, and 18–19% in sporadic cases (33). The frequency of *p53* mutations and protein overexpression in breast tumors from *BRCA2* mutation carriers is intermediate between *BRCA1* mutation carriers and nonhereditary cancers, and has not always been statistically different from the latter (31,66,67). Mutations in *p53* occur at extremely high frequencies in typical medullary breast cancers (68), an observation of particular interest in view of the recognized pathobiologic similarities between medullary and *BRCA1*-associated breast cancers (69). Moreover, *BRCA1/2*-associated breast cancers carry novel *p53* mutations not detected in control series of cancers, suggesting that the spectrum of *p53* mutations occurring in *BRCA1/2*-associated tumors is distinct from that in sporadic cases (64). Since p53 interacts physically with BRCA1 and BRCA2, it was suggested that the presence of a *BRCA1/2* mutation could be the determining factor in the selection of these mutations in breast cancer (70–72).

Nevertheless, the precise biological and clinical consequences of this increased frequency of *p53* mutations in *BRCA1/2*-associated breast cancer cases are elusive. The BRCA1 and BRCA2 have been shown to interact with the Rad51, which is involved in recombination and DNA double-strand repair (15,17). A cell lacking BRCA1/2 may have a decreased ability to repair DNA damage, which would lead to genomic instabil-

ity. Functional p53 would prevent this abnormality, through either cell-cycle arrest or apoptosis. In the absence of functional p53 gatekeeper, the clonal population of cells could continue to proliferate and accumulate further somatic genetic abnormalities, resulting in tumor progression. Thus, loss of p53 checkpoint control may be obligatory for malignant transformation in cells with *BRCA1/2* mutations (73). This hypothesis is supported by data from murine models demonstrating that loss of p53 led to accelerated tumor formation, increased tumor incidence, and striking genomic imbalances in *BRCA1/2*-deficient animals (52,74–76). Armes et al. (31) were able to show that p53 overexpression consistently occurred at the preinvasive (ductal carcinoma *in situ* [DCIS]) stage of disease and suggested that p53 overexpression is an important and early event in the molecular pathogenesis of cancers arising in *BRCA1/2* mutation carriers. All these findings may have prognostic significance because breast carcinomas with p53 mutations tend to have a worse prognosis than those without (77). Although *tp53* mutations occur more often in *BRCA1/2*-associated tumors than in sporadic breast cancers, no absolute requirement for p53 mutation has been proven (52,65).

Some studies have not found the frequency of p53 alterations to be different from that found in sporadic breast cancers as measured by IHC (34,42,66). An analysis of a large data set of *BRCA1/2*-associated breast cancers compared with control cancers revealed a complex pattern of p53 immunostaining (36). The proportion of tumors showing strong p53 staining was higher in *BRCA1*-associated tumors than in controls, as was the proportion with staining in >50% of cells (44 vs 22%). The proportion with weak staining was actually lower in *BRCA1* carriers, however, and the authors were not able to show clear relationship between p53 staining and *BRCA1* status.

Further evidence for an important role for p53 in familial breast cancer is needed. If loss of the wild-type *BRCA* allele and loss of the p53 pathway are both required for tumorigenesis, it is not known whether loss of the p53 pathway precedes or is followed by the loss of the normal allele of the *BRCA* gene (6,78). In either case, other mutations, perhaps those activating dominant oncogenes, would then take place because of the mutagenic environment caused by the genomic instability driven by the mutant *BRCA* protein.

It is well established that the loss of the E-cadherin/catenin cell-adhesion complex promotes tumor progression and metastasis in many cancers. *E-cadherin* is a TSG on 16q22.1. It is a transmembrane cell–cell adhesion molecule, which has an essential role in the generation and maintenance of epithelial cell polarity. *E-cadherin* is mutated at a high frequency in invasive lobular carcinomas (79,80), which have been shown to have links to familial breast cancer. Studies indicate, however, that *E-cadherin* alterations (germline and, more commonly, somatic mutations, and promoter methylation) occur at a low frequency in familial breast cancer (81). No evidence exists that *E-cadherin* mutations predispose women to breast cancer or are involved in *BRCA1/2* phenotypes. Although no obvious differences between *BRCA1/BRCA2* and control groups of breast cancers have been noted in the immunophenotypes of the anti-E-cadherin or  $\beta$ -catenin studies to date (31, 82),  $\beta$ -catenin was expressed more frequently ( $p = 0.05$ ) in tumors of *BRCA1* mutation cases than in matched controls diagnosed before age 50 (82).

## **Oncogenes**

### **c-MYB**

*c-MYB* is located on 6q, where high regional copy number gain at 6q22–24 was observed in one *BRCA1*-mutated tumor (83). *c-MYB* is a member of the vertebrate



*MYB* gene family, which encodes DNA-binding proteins that regulate transcription and have been implicated in the regulation of the cell cycle, affecting cell proliferation, differentiation, and apoptosis (84–86). In addition to a critical role in hematopoiesis and erythroid cell differentiation, MYB may promote tumor development in a variety of cancers including lung, colorectal cancer, neuroblastomas, and breast epithelium (86). *c-MYB* is one of the factors related to an increased resistance of colon cancer to cisplatin (87). MYB proteins are crucially involved in the processes of DNA recombination and mammary gland development (88). *c-MYB* may have a role in hormone-regulated growth and differentiation of breast epithelial cells and ER-positive breast cancers. Kauraniemi et al. (83), using CGH, DNA microarray, and FISH, revealed *c-MYB* oncogene amplification in 5 of 17 (29%) *BRCA1*-associated breast tumors, whereas none of the 8 *BRCA2*-associated tumors and 13 breast cancer cell lines, and only 2/100 (2%) sporadic breast tumors, exhibited altered *c-MYB* copy number. Gene amplification resulted in mRNA and protein overexpression. These researchers concluded that *c-MYB* amplification is infrequent in sporadic breast cancer but common in breast tumors from *BRCA1* mutation carriers, suggesting a role for *c-MYB* in the progression of some *BRCA1*-mutated tumors (83). This finding is surprising in light of a number of existing publications postulating that *c-MYB* expression in breast cells is associated with estrogen stimulation and the presence of ER (89,90). It has been suggested that because *BRCA1* tumors usually are ER-negative, the activity of *c-MYB* (single or amplified copy number) in *BRCA1*-deficient cells would be regulated by other pathways. Possibly, *c-MYB* amplification in *BRCA1*-mutated tumors may reflect a compensatory mechanism to execute vital cellular functions or to override a cell-cycle block caused by DNA damage. The researchers, however, could not rule out the significance of other genes neighboring *C-MYB* in the 6q22–24 amplicon. Determining whether this novel amplified region and, in particular, *C-MYB* oncogene are essential in hereditary cancer will have to await more studies on a larger set of tumors.

## TBX2

The *TBX2* gene on 17q23 (91) has been shown to be involved in the complex multi-gene amplicon formation on 17q22–24 (92–94). As discussed earlier, this region is amplified not only in breast cancer cell lines, primary breast cancers, and other solid tumors (95,96), but is frequently amplified in hereditary breast cancers (41). The selection of 17q22–24 for gain in *BRCA1*- and *BRCA2*-associated breast cancers suggests that a gene(s) localized to this region is important in the progression of hereditary breast tumors (41). *TBX2* appeared to be preferentially amplified within 17q22–24 in hereditary breast cancers (97). The FISH-detected amplification frequency of *TBX2* gene (from this amplicon) was compared in 27 *BRCA1*- and *BRCA2*-mutant breast tumors, 15 breast tumors from high-risk patients with no *BRCA1/2* mutations, and 62 matched sporadic controls (97). *TBX2* was more frequently amplified (ratio  $\geq 1.5$ ) in *BRCA1/2*-associated tumors (70%) than in the matched sporadic controls (22%,  $p = 0.0001$ ). Similarly, an amplification ratio  $\geq 2.0$  was found in 8/27 (30%) *BRCA1/2* tumors but in only 3/37 (8%) sporadic controls ( $p = 0.04$ ). The proportion of *TBX2*-amplified tumors from patients with familial breast cancer who were negative for *BRCA1/2* mutations was similar to matched sporadic controls. *TBX2* overexpression was highly correlated with gene amplification (92,93,97). *TBX2* was amplified equivalently in DCIS and invasive tumors, suggesting that it might be an early event in tumor progression.

These data suggest that *TBX2* may be the target gene driving amplification of the 17q22–23 region in *BRCA1/2*-associated tumors, and contributes to the initiation and/or progression of *BRCA*-associated hereditary cancers, but other genes should not be excluded (97). *TBX2* is a member of the T-box family of phylogenetically conserved transcription factors, DNA-binding proteins, which are known to regulate gene expression during key developmental events. Expression studies of the T-box genes in vertebrates revealed highly tissue-specific patterns of expression during embryogenesis and organogenesis (98). Mutations in some members of the T-box family have been linked to human disease and birth defects (99). For *TBX2*, a potential role in breast cancer development has been predicted because *TBX2* may assist early stage tumor cells in bypassing senescence. It has also been suggested that *TBX2* may contribute to immortalization, proliferation, and neoplastic transformation through its downregulation of the p19<sup>ARF</sup>/MDM2/p53 pathway (92,99,100). If unique amplification of *TBX2* in *BRCA1*- and *BRCA2*-associated tumors is confirmed on a larger group of hereditary breast cancers, the *TBX2* oncogene may be a useful predictive/prognostic marker in familial breast cancer.

### c-MYC

The *c-MYC* oncogene encodes a proliferative nuclear DNA-binding protein, deregulated expression of which has been shown to play an important role in the induction and progression of lymphomas, lung cancer, and breast cancer (101–103). A number of studies have reported amplification and/or overexpression of *c-MYC* in sporadic breast cancers, ranging from 5–50% of cases studied (102). *c-MYC* amplification/overexpression has been shown to associate with poor prognostic factors such as high tumor grade, ER-negativity, and high proliferation rate, which are also characteristic of *BRCA1*-mutated cancers (102). Therefore, in our laboratory we addressed the contribution of *c-MYC* to human hereditary *BRCA1*-mutated and *BRCA1*-methylated sporadic breast cancers (104,105). Using a *MYC/CEP8* FISH assay on formalin-fixed paraffin-embedded tumor tissues from 28 women with known deleterious germline *BRCA1* mutations and 49 sporadic cases, we showed *c-MYC* amplification in both *BRCA1*-associated and sporadic tumors. We found that 13/49 (27%) of sporadic tumors had a *MYC:CEP8* amplification ratio of  $\geq 2$ , a proportion of amplified tumors comparable to the approx 25% of breast tumors that have been reported with *c-MYC* amplification in the literature (102). In the *BRCA1*-mutated group, however, the proportion of *c-MYC*-amplified tumors was significantly higher (17/28, [61%],  $p = 0.004$ ). In a multivariable regression model, controlling for age, tumor size, and ER status, *BRCA1*-mutated tumors demonstrated significantly greater *MYC:CEP8* ratios than sporadic tumors ( $p = 0.05$ ).

These data suggest that the loss of *BRCA1* in hereditary breast cancers is associated with *c-MYC* amplification and that the aggressive features of *BRCA1*-associated tumors are in part due to dysregulated *c-MYC* oncogenic activity. Our results are consistent with data from DNA microarray studies. A review of the set of genes published by Hedenfalk et al. (106) and a recent article by van't Veer et al. (107) show that *c-MYC* on 8q24 was overexpressed in *BRCA1* mutation carriers. Our data are supported by studies in mouse models of carcinogenesis. Mice carrying a conditional *BRCA1* mutation display gain of chromosome 15 (orthologous to human chromosome 8q24) by CGH and overexpression of *c-MYC* protein by Western blot analysis (53,108). *BRCA1* protein structure (nuclear localization sequences and transactivation domain) suggests that *BRCA1* may be involved in gene transcription. Consistent with this notion, Wang et al.

(109) showed that BRCA1 physically binds to c-MYC and represses its transcriptional and transforming activity. Furthermore, they showed that BRCA1 reverses the phenotype of rat embryonic fibroblasts transformed by *c-myc-ras* activation. Another group found that in addition to direct binding to c-MYC, BRCA1 specifically binds to Nmi (N-MYC-interacting protein), which functions as an adapter molecule to recruit c-MYC to a complex with BRCA1 (110). These investigators showed that constructs carrying *BRCA1* mutations within Nmi-binding sites are unable to suppress the oncogenic potential of c-MYC through disruption of Nmi-BRCA1-MYC tricomplex. These data indicate that BRCA1 is a component of a transcription factor complex and may in part function as a tumor suppressor by regulating MYC activity (110). Therefore, loss of *BRCA1* in germline mutation carriers may result in increased c-MYC activity and transforming potential, which, in turn, can lead to gene amplification through abnormal auto-regulation affecting its DNA replication (102,111,112).

Further studies of *c-MYC* amplification and overexpression on a larger cohort of *BRCA1* mutation carriers are necessary to confirm our observation that c-MYC activation occurs in a high proportion of human *BRCA1*-mutated hereditary (and *BRCA1*-methylated sporadic) cases, possibly providing further support for a role for *c-MYC* in aggressive multistep tumor progression in hereditary *BRCA1*-mutated tumors. *c-MYC* status in *BRCA2*-associated tumors has not been reported.

#### HER-2/neu and Ki-ras

*HER-2/neu* (*HER2*, *ERBB2*) encodes a 185-kDa transmembrane cell-surface receptor glycoprotein with tyrosine kinase activity that belongs to the epidermal growth factor receptor (EGFR) family (113). The *HER-2/neu* protooncogene is involved in the regulation of normal cell growth and division and is expressed at low levels in many normal epithelial cells. The *HER-2/neu* oncogene is associated with tumor aggressiveness and enhanced chemoresistance of cancer cells through the mechanism of gene amplification, followed by increased transcription and higher levels of protein expression. A high level of HER-2/neu protein expression is associated with accelerated cell growth and proliferation. *HER-2/neu* amplification/overexpression has been reported in 20–30% of human breast cancers (114) and in varying degrees in tumors of the ovary, endometrium, and other organs (113). Breast cancers with *HER-2/neu* amplification/overexpression are biologically aggressive and are associated with an increased risk of disease recurrence and shortened overall survival. In multiple studies, *HER-2/neu* amplification/overexpression has been shown to be an independent prognostic and predictive marker of response to targeted therapy with trastuzumab (115–117).

The *HER-2/neu* oncogene and the *BRCA1* TSG are located close to each other at 17q11–12 and 17q12–21, respectively. Both *BRCA1*-associated and *HER-2/neu*-amplified tumors occur in a subset of young women with histologically aggressive, hormone receptor negative, and highly proliferative breast cancers, suggesting a contribution of *HER-2/neu* oncogene to *BRCA1*-associated tumor aggressiveness. Several studies attempted to clarify this association, using small populations of *BRCA1* germline mutation carriers. Their results suggested lower incidence of HER-2/neu overexpression among *BRCA1*-associated cases compared with control cases (31,42,66). This particular feature of *BRCA1*-related breast cancer was confirmed by a larger study in our laboratory (118). We performed molecular cytogenetic (FISH) analysis of the *HER-2/neu* gene in 53 *BRCA1*-associated breast cancers and showed that high levels of the *HER-2/neu* oncogene amplification do not occur in breast tumors from *BRCA1* germline mutation

carriers. By contrast, *HER-2/neu* was highly amplified in 6/41 (15%) of sporadic breast tumors a result consistent with the findings of other investigators (119,120). We also showed by IHC that most nonamplified *BRCA1*-associated and sporadic tumors were negative for *HER-2/neu* protein expression, but that high amplification of *HER-2/neu* in sporadic tumors was invariably accompanied by a high level of protein expression. The results of our FISH study were confirmed by a large collaborative study done on behalf of the Breast Cancer Linkage Consortium (36). Samples of breast cancers from >100 *BRCA1* mutation carriers were characterized by IHC and were *HER-2/neu* negative. Only 3% of *BRCA1*-mutated cases were positive for *HER-2* protein expression, compared with 15% of sporadic cases. In a DNA microarray study comparing *BRCA1*- and *BRCA2*-associated tumors with sporadic tumors, Hedenfalk et al. (106) demonstrated that in addition to several genes that are differentially expressed or downregulated, *HER-2/neu* overexpression was not observed in *BRCA1*-associated tumors.

CGH analysis has previously shown different gains and losses in *BRCA1*-associated tumors (3p, 3q, 5q, and 12q losses; 6p, 10p, and 17q22–24 gains) (41,46) versus *HER-2/neu*-amplified breast carcinomas (18q losses; 20q and 17q11–21 gains) (121). Therefore, it is reasonable to conclude that *HER-2/neu* amplification/overexpression is not a feature of *BRCA1*-associated tumors, and that trastuzumab is unlikely to play a role in the management of breast cancer in patients harboring mutations in *BRCA1*.

Although some theories have been proposed, no known mechanism explains why *HER-2/neu* is never highly amplified or overexpressed in the background of a *BRCA1* germline mutation (118). *HER-2/neu* and *BRCA1* may participate in distinct molecular pathways. It is reasonable to postulate that once early inactivation of the normal *BRCA1* allele and activation of a specific oncogene(s) occurs (41,83,97,104, 105) in the breast tissue of germline *BRCA1* mutation carriers, there is little selection pressure for the recruitment of *HER-2/neu* amplification (31). Alternatively, the *BRCA1* locus on chromosome 17 could potentially have a role in controlling amplification at the *HER-2/neu* locus. Further dissection of these pathways may allow elucidation of the conditions required for *HER-2/neu* amplification in human breast cancer cells. A better understanding of the molecular mechanisms underlying the mutually exclusive functions of *BRCA1* and *HER-2/neu* will lead to the rational design of effective therapeutic targets against these biologically aggressive breast cancers, which disproportionately affect young women.

The role of *HER-2/neu* in *BRCA2*-associated breast cancer is unclear at present. Analysis of *HER-2/neu* gene amplification has not been reported. Studies that have examined *HER-2/neu* overexpression by IHC reported either low frequency of *HER-2/neu* overexpression in *BRCA2* tumors similar to that of *BRCA1* cases (36,63,66), or frequent *HER-2* positivity in *BRCA2*-associated tumors that was comparable to that of sporadic controls (31,33,67).

*K-RAS* gain-of-function mutations confer growth advantage and were expected to be frequent in *BRCA1/2*-deficient cells. Mutation analysis by Crook et al. (61), however, did not reveal a frequency of mutations in *BRCA1/2*-associated tumors above that observed in sporadic breast tumors of similar grade.

### **Cell-Cycle and Apoptotic Proteins**

If *BRCA*-linked disease requires inactivation of checkpoint gene(s) followed by a *BRCA* gene loss, then the tissue specificity of *BRCA*-linked disease might arise from a specific predisposition of the breast (and ovarian) epithelium to lose the function of

such checkpoints (10). Checkpoint loss is a necessary precursor of *BRCA1/2* gene inactivation in tumorigenesis. Most cells that have inactivating mutations of *BRCA* will be unable to repair DNA damage sustained in the following cell cycle and will die. In the rapidly proliferating breast epithelium, some repair-deficient cells may escape death, at least briefly. Because these *BRCA*-null cells are deficient in repair, they would sustain DNA damage at many sites, often including genes essential to cell-cycle checkpoint activation. Mutation of a checkpoint gene would enable a *BRCA*-null cell to escape death permanently and to proliferate. Therefore, genetic instability caused by loss of *BRCA1/2* may enable the accumulation of additional mutations, including alterations in checkpoint genes. Alterations in p53, one of the key checkpoint genes, appear to be crucial. Cells that have successfully escaped death at checkpoints will accumulate multiple mutations and/or alterations in proteins controlling the cell cycle and apoptosis.

p16 (INK4a/CdkN2A) is an inhibitor of Cdk4/D cyclins. It functions to inhibit the phosphorylation and inactivation of pRb by Cdk4/D cyclins. The normal p16 protein maintains cell-cycle arrest. A nonfunctional p16 protein has lost its regulatory capacity and cannot constrain cells from passing through the cell cycle (122). Inherited mutations in *p16* TSG and in *Cdk4* confer susceptibility to cutaneous malignant melanoma and an increased risk of breast cancer in *CdkN2A*-associated melanoma families (123). *p16* has been proposed as a multiple TSG and has been suggested by one group to be a breast cancer susceptibility gene (124). The frequencies of downregulation or loss of *p16* detected in familial tumors were similar to those seen in sporadic breast tumors (61,125–127).

The cyclin-dependent kinase (CDK) inhibitor p21 (Cip1) blocks transition from G1- to S-phase and suppresses cell proliferation. p21 is thought to be a major downstream effector of the wild-type p53-mediated growth arrest pathway that is induced by DNA damage. In sporadic breast tumors, the expression of p21 is inversely correlated with p53 expression and high grade (32,128), apparently owing to the inability of mutated p53 to activate p21 transcription. In *BRCA1*-associated tumors, expression of p21 was detected in 9/26 (35%) tumors analyzed, these comprising four wild-type and five mutants for p53. Three tumors wild-type for p53 did not express p21 despite having high levels of p53 protein (61). Similarly, in *BRCA2*-associated tumors, p21 was expressed regardless of p53 status, and, some tumors expressing wild-type p53 did not express detectable p21 (61). No obvious differences between hereditary and sporadic breast cancers were noted in the immunophenotype of p21 (31). Thus, IHC studies have failed to show a relationship between p21 and p53 in *BRCA* tumors, suggesting that p21 transactivation in hereditary tumors could be mediated by a p53-independent mechanism. This finding could be of practical significance, because an increase in p21 expression and apoptosis has been observed in cells with wild-type p53 exposed to chemotherapy (129).

Another CDK complex inhibitor that plays an important role in breast cancer pathogenesis is p27 (Kip1). In normal cells, p27 expression is crucial for the G1- to S-phase transition. p27 was demonstrated to bind to cyclin E/Cdk2 complexes and inhibit the kinase function of Cdk2 (130). Several other functions have been suggested for p27, including being a promoter of apoptosis, a regulator of drug resistance in solid tumors, and having a role in cell differentiation (131). Decreased levels of p27 in breast cancer are associated with a poor outcome. Patients whose tumors overexpress p27 have significantly higher survival rates (32). In small breast cancers (stages T1a and b), p27 expression was reported as the only independent prognostic factor (132). Data regard-

ing p27 expression, as well as that of other cell-cycle proteins, in familial *BRCA1/2*-associated breast cancer are limited and contradictory. p27 expression does not differ between sporadic (33/40 [83%]) and *BRCA*-associated (15/16 [94%],  $p = 0.28$ ) breast cancers in one study (66). This finding is contrary to other observation (32), in which p27 was overexpressed in *BRCA1/2* breast cancers (86% in familial tumors vs 65% in sporadic tumors). In a comprehensive study of 202 consecutive Ashkenazi Jews by Chappuis et al. (33), 32 (16%) tumors were positive for *BRCA1/2* mutations. Low p27 expression was seen in 110 (63%) tumors and was significantly associated with *BRCA1/2* mutations ( $p = 0.009$ ). *BRCA1/2* mutation carriers had a significantly worse 5-yr distant disease-free survival compared to women without *BRCA1/2* mutations ( $p = 0.003$ ). Similar results were seen for women whose tumors expressed low levels of p27, compared with those with high levels ( $p < 0.0001$ ). In a multivariate analysis, both *BRCA1/2* mutations and low p27 expression were associated with a shorter distant disease-free survival ( $p = 0.05$  and  $p = 0.01$ , respectively). In addition, decreased p27 protein expression was observed in the *BRCA1*-mutant cell line HCC1937 compared with MCF-7 and other breast cancer cell lines expressing intact BRCA1 protein (133,134). It was concluded that *BRCA1/2* mutations are associated with low levels of p27 in breast cancer, and that both *BRCA1/2* and p27 status can be identified as independent prognostic factors.

Recently, it was shown that BRCA1 transactivates expression of p27 through direct interaction with p27 promoter, and that the transcriptional regulation of p27 by BRCA1 may be a mechanism of BRCA1-induced growth inhibition (134). At the same time, as we noted above, it was shown that BRCA1 binds to c-MYC and represses its transcriptional and transforming activity (109). c-MYC in turn binds to p27 and represses p27 promoter activity (135). Therefore, p27 regulation by BRCA1 can be direct or indirect through c-MYC. In *BRCA1*-deficient cells, therefore, one would expect upregulation of c-MYC and downregulation of p27. Indeed, we observed *c-MYC* amplification in a significant proportion of *BRCA1*-deficient cells (104,105), and downregulation of p27 was found in an even higher proportion of *BRCA*-mutated tumors (33).

The recent concomitant finding by several research groups (136–138) that p27 cytoplasmic relocalization rather than downregulation might be a mechanism of p27 alteration in breast cancer may prompt more rigorous analysis of p27 expression in hereditary breast cancer and may eliminate discrepancies among existing results. Understanding the mechanisms controlling p27 expression in hereditary breast cancer may provide new strategies to inhibit tumor growth. p27 may have greater clinical utility in *BRCA*-associated cancer prognosis than other candidate markers.

In hereditary breast cancer, one would expect an inverse correlation between loss of p53 expression/high proliferative index, and low expression of the antiapoptotic gene *Bcl-2*, as has been demonstrated for sporadic tumors (32). Indeed, reduction of expression the anti-apoptotic gene *Bcl-2* was a characteristic of most breast tumors from *BRCA1* mutation carriers (139). Two early studies have shown that *BRCA*-deficient tumors have the same levels of *Bcl-2* expression as the control groups, despite being highly proliferative and having frequent p53 mutations (31,66).

IHC analysis of cyclin D1 expression gave controversial results. In one study, the proportion of cases expressing cyclin D was 1/10 (10%) in *BRCA1*-positive tumors and did not show a statistically significant difference with the control group (15/43 [35%];  $p = 0.25$ ) (140). Robson et al. (66) reported that the proportion of *BRCA* mutation-positive cases was 6/12 (50%) and was similar to mutation-negative cases (15/32

[47%];  $p = 0.85$ ). In another study, only 1/9 (11%) cancers in *BRCA1* mutation carriers showed strong or moderate staining of cyclin D1 that was significantly lower, compared with 12/19 (63%;  $p = 0.02$ ) of control cancers (31). The same group reported that the frequency of cyclin D1 expression in *BRCA2* mutation carriers was intermediate between *BRCA1* mutation carriers and control cancers (31). A study by Osin et al. (28) also detected a significantly lower frequency of cyclin D1 expression in *BRCA1*-mutated tumors (1/21 [5%]) than in *BRCA2*-associated (4/15 [27%];  $p < 0.05$ ) or in sporadic tumors (24/69 [35%];  $p < 0.01$ ). These results were confirmed by cDNA microarrays followed by a tissue array study of *BRCA1/2*-associated breast cancers (106). Recent FISH and IHC analyses revealed that *cyclin D1* is amplified in 19/74 (26%) of sporadic breast tumors but is not amplified or showed low frequency of protein expression in tumors of *BRCA1* mutation carriers diagnosed before age 50 yr (82,141). However, high frequency of cyclin D1 expression was detected in *BRCA1* tumors diagnosed at age 50 yr or older and was comparable to matched controls. Based on the analysis of cyclin D1 and other markers, the authors concluded that breast tumors of *BRCA1* mutation carriers diagnosed with breast cancer before the age of 50 yr display a distinct tumor phenotype compared with age-matched controls, and that tumors of carriers diagnosed at age 50 yr or older display a phenotype that is similar to that of age-matched sporadic breast cancers. The authors suggested that the breast tumor phenotype of *BRCA1* mutation carriers may be influenced by age at diagnosis. Cyclin D1 is a regulator of progression from G1- to S-phase in the cell cycle and is known to be upregulated by estrogen and progestins, and downregulated by antiestrogens (142). The transcription of ER-regulated genes is modulated by cyclin D1 (32). Thus, it is not surprising that there could be an age-related relationship between the ER and PR of *BRCA1*- and *BRCA2*-associated tumors.

Cyclin E expression has been implicated as a potential prognostic marker for breast cancer (143). No significant differences were observed in tumors of *BRCA1* mutation carriers compared with age-matched controls (82). Data on *BRCA2* tumors have not been reported.

Interestingly, in a *BRCA1* conditional mouse model, the expression of cell-cycle regulators p16 and cyclins A, B1, and E was notably absent, but cdc2, p21 (weak), cyclin D1, and p27 were present (108). Disruption of *BRCA2*, in contrast to *BRCA1*, does not appear to have a marked effect on cell-cycle checkpoint enforcement. Instead, *BRCA2*, along with the BubR1 kinase, inactivates the metaphase-to-anaphase surveillance mechanism, reverses proliferative arrest and fosters tumorigenesis in *BRCA2*-deficient cells (144,145). Mitotic checkpoint inactivation in *BRCA2*-deficient animals was accompanied by increased expression of p53 and p21 (53,75,144).

### **Other Markers and Genes Involved in Metabolism of Steroid Hormones**

Human mammary glands contain two types of epithelial cells, basal and luminal, which can be easily distinguished by the patterns of expression of certain cytokeratins. The cytokeratins are a family of approx 20 cytoskeletal intermediate filamentous proteins (146). Basal-like breast epithelial cells express exclusively basal keratins 5 and 17 and vimentin, while luminal cells express mainly the luminal cytokeratins 8 and 18. The cytokeratin pattern is largely conserved after transformation of epithelial cells; therefore, the cell-type origin of primary carcinoma can be determined. Most breast cancers originate from luminal epithelium and express luminal cell-specific cytokeratins. Three to 15% of all breast cancers originate from basal-like epithelium, express basal-specific

cytokeratins, and represent a more aggressive group of tumors. Tumor cell-type origin and patterns of cytokeratin expression have been implicated in the prognosis of breast carcinoma (146). Perou et al. (147, 148), used cDNA microarrays followed by IHC, in an attempt to classify breast cancers based on variations in gene expression patterns. They found that breast cancers encompass at least three biologically distinct subtypes of tumors: ER-negative/basal-like tumors that are positive for keratins 5 and 17, ER-negative/basal-like/HER-2-positive tumors, and ER-positive/luminal-like tumors that are positive for cytokeratins 8 and 18. In collaboration with Perou, we conducted a preliminary study of cytokeratins in *BRCA1*-associated tumors. Six of seven tumors with *BRCA1* mutations that we have examined to date revealed positive staining for basal keratin 5, basal keratin 17, or both (149). Taking into account that *BRCA1*-associated tumors are negative for HER-2/neu and are ER negative (36,118), these findings are compatible with the ER-negative/basal-like pattern of gene expression. Consistent with our observation, Hedenfalk et al. (106) showed that the level of expression of cytokeratin 8 was low in tumors with *BRCA1* mutations, whereas HER-2/neu was not overexpressed. By contrast, cytokeratin 8 was highly expressed in tumors with *BRCA2* mutations, a pattern of expression described for ER-positive sporadic tumors of luminal origin. Thus, cytokeratins alone or in combination with an accompanied set of genes (106,147) can be potential markers of the cellular origin of *BRCA1*-associated tumors and prognosis.

Cathepsin D belongs to a family of proteases that are involved in tissue remodeling. Overexpression of cathepsin D in breast cancers has been found to correlate with poor prognosis (150). The expression of cathepsin D in host stromal cells is associated with higher intratumoral microvessel density. Expression of cathepsin D in *BRCA1/2*-associated breast cancers was not found to be different from that in sporadic breast cancers (31,66).

Genes involved in the metabolism of steroid hormones have been hypothesized to modify breast or ovarian cancer risk in carriers of *BRCA1/2* mutations. The growth regulatory effects of bioavailable steroid hormones may be modified by inherited genotypes at numerous loci including *CYP11A1*, *CYP3A4*, *CYP17*, or *CYP19*, as well as AR and ER (151). A study by Rebbeck et al. (152) showed that *A1B1* genotype and reproductive history may affect *BRCA1/2*-associated breast cancer risk, and supported the hypothesis that pathways involving endocrine signaling may modify cancer risk in mutation carriers. The role of steroid hormones in *BRCA1/2*-mediated carcinogenesis has not been completely elucidated (153–155).

### **Gene Expression Profiles of *BRCA1*- and *BRCA2*-Associated Breast Cancers**

Great efforts have been made to identify single molecular markers for breast cancer, especially hereditary breast cancer, which would have a large impact on both estimation of prognosis and the choice of therapy for the individual patient. The results have been unimpressive so far. cDNA microarrays offer a systematic method to perform very extensive expression profiling within a single cancer specimen. Two studies aimed at identifying the distinct patterns of gene expression in *BRCA1*- and *BRCA2*-associated breast carcinomas have been published (106,107). In a study by Hedenfalk et al. (106), RNA from samples of primary tumors from seven *BRCA1* mutation carriers, seven *BRCA2* mutation carriers, and seven patients with sporadic cases of breast cancer were compared, using a microarray of 6512 DNA clones of 5361 genes, including 2905 known genes. The *BRCA1*- and *BRCA2*-associated tumors generally had dispar-



ate histologic patterns and hormone receptor status. The investigators identified 51 genes that differentiated most clearly among the three types of tumors, using a modified F test ( $\alpha = 0.001$ ). In addition, they used a class-prediction method to determine whether the gene expression profiles of the 22 breast-tumor samples accurately identified them as positive or negative for *BRCA1* mutations or for *BRCA2* mutations. For the analysis of all 22 tumor samples, nine genes were differentially expressed between *BRCA1* mutation-positive tumors and *BRCA1* mutation-negative tumors. The nine genes were *KRT8*, *VLDLR*, *MCM7*, *RSTs* (*Hs.239666*), *HECH*, *ESTs* (*Hs.91604*), *BRF1*, *TP53BP2*, and *SPS*. Eleven genes were differentially expressed between *BRCA2* mutation-positive tumors and *BRCA2* mutation-negative tumors: *ARPI*, *PCNA*, *HADHA*, *INTB8*, *PPP1CB*, *D123*, *CDK4*, *UGTREL1*, *ZNF161*, *ARVCF*, and *PDGFB*. All seven tumors with *BRCA1* mutations and 14 of 15 tumors without *BRCA1* mutations were correctly identified in the *BRCA1* classification. Five of 8 tumors with *BRCA2* mutations and 13 of 14 tumors without *BRCA2* mutations were correctly identified in the *BRCA2* classification. Thus, the accuracy of classification was significant for the identification of *BRCA1* mutation-positive tumors and less significant for the identification of *BRCA2* mutation-positive tumors.

Using three statistical methods, Hedenfalk et al. (106) identified 176 genes that are most important in distinguishing a *BRCA1* mutation-positive breast cancer from a *BRCA2* mutation-positive breast cancer. Within the list is a large block of genes that are upregulated in *BRCA1*-mutation positive samples but not in *BRCA2* mutation-positive samples. Examination of individual genes in this block suggests the coordinated transcriptional activation of two major cellular processes in *BRCA1* mutation-positive samples: DNA repair and apoptosis. DNA repair pathways are reflected by genes (e.g., *MSH2*) that participate in the activation of cellular responses to stress. In addition, *BRCA1* mutation-positive tumors display increased expression of genes associated with inducing apoptosis (e.g., *PDCD5*) and decreased expression of genes involved in suppressing apoptosis (e.g., *CTGF*). The investigators suggested that the mutation of *BRCA1* leads to a constitutive stress-type state. Using high-density tissue microarrays, they showed that levels of proteins encoded by selected genes (measured by IHC) correlated with the cDNA microarray results. Thus, data from this DNA microarray study (106) suggest that breast cancers arising in the setting of germline *BRCA1/2* mutations have unique gene expression profiles, and it can be concluded that a heritable mutation influences the gene expression pattern of the cancer.

Another group, van't Veer et al. (107), performed genomewide gene expression profiles of 18 *BRCA1*-associated tumors and 2 *BRCA2*-associated tumors, as part of a larger study of 117 primary breast cancers aimed at identifying a set of genes to predict disease outcome and developing a strategy to select patients who would benefit from adjuvant therapy. Microarray data were evaluated for approx 25,000 human genes and analyzed using an unsupervised hierarchical clustering algorithm. Approximately 5000 genes appeared to be significant in the tumor clustering, and, on the bases of these genes, tumors were divided into two groups: those with good prognosis and those with poor prognosis. The group of poor-prognosis tumors had a dominant expression signature including down-regulated ER $\alpha$  gene (*ESR1*) and ER-targeted genes. A second dominant gene cluster was associated with lymphocytic infiltration and included several genes expressed primarily by B- and T-cells. It was shown that the poor-prognosis signature consists of genes regulating cell cycle, invasion, metastasis, and angiogenesis. In addition, the investigators established a signature that identifies tumors of *BRCA1* mutation

carriers. Sixteen of 18 tumors of *BRCA1* carriers were found within the poor-prognosis group, which was consistent with the idea that most *BRCA1*-mutant tumors are ER-negative and manifest a higher degree of lymphocytic infiltration. This finding is in contrast to that of the Hedenfalk et al. (106) study, which was unable to appreciate the overlap in signatures between the ER-negative and *BRCA1*-mutated tumors. The two tumors of *BRCA2* carriers, however, were a part of the group with good prognosis and did not show similarity with *BRCA1*-mutated tumors, which is consistent with the conclusion by Hedenfalk et al. (106) that *BRCA1*- and *BRCA2*-associated tumors have different gene expression profiles. van't Veer et al. (107) identified an optimal set of 100 genes that can distinguish *BRCA1* tumors from sporadic cases. This set is enriched in lymphocyte-specific genes. The nine *BRCA1* status reporter genes listed in Hedenfalk et al. (106) publication were not present in this set of 100 genes. Different data analysis and a different set of hereditary tumors use of may account for these discrepancies.

Nevertheless, both groups showed that gene expression profiles are a more powerful predictor of the outcome of disease in young patients with breast cancer than the standard system, based on clinical and histologic criteria (107,156). They also showed that genotype–phenotype correlations exist, and that the signature that reveals *BRCA1* status may further improve the diagnosis of hereditary breast cancer (106). Another important finding from both studies is that sporadic tumors with methylated *BRCA1* may be misclassified within the *BRCA1* mutation–positive group. This suggests that *BRCA1*-methylated sporadic tumors display pathologic features and gene expression profiles similar to that of *BRCA1*-mutated hereditary breast cancers, and that promoter methylation may be an important mechanism for functionally inactivating *BRCA1* in nonhereditary forms of breast cancer. These findings are consistent with our study of *c-MYC* amplification in *BRCA1*-deficient breast cancers (*BRCA1*-mutated hereditary and *BRCA1*-methylated sporadic), in which we showed that the pattern of *c-MYC* amplification in *BRCA1*-methylated cases is more similar to those of *BRCA1*-mutated cases rather than to those of sporadic cancers (104,105).

## Epigenetic Lesions in Hereditary Breast Cancers

Aberrant methylation of single or multiple genes was suggested as a marker for detection of cancer cells, for tumor behavior, and as a target for therapy (157). *BRCA1* itself undergoes CpG island hypermethylation in a subset of sporadic breast tumors (157), whereas *BRCA2* does not (158). The impact and significance of the epigenetic silencing of *BRCA1* is functionally equivalent to carrying a germline *BRCA1* mutation. Both events lead to the same disturbance of gene expression in a cancer cell (104–107). Epigenetic changes in cancer are not limited to hypermethylation of gene promoter CpG islands; they also include a simultaneous global demethylation of the genome (159). Total DNA hypomethylation of the malignant cell has been proposed as a cause for chromosomal instability, reactivation of endogenous viral sequences, and upregulation of certain genes (160). Total levels of methylation in both inherited and sporadic forms of breast cancer have been studied by Esteller et al. (127) to compare epigenetic processes in these alternate pathways of cancer development. This study examined CpG island methylation of 10 genes (*hMLH1*, *BRCA1*, *APC*, *LKB1*, *CDH1* [*E-cadherin*]), *p16<sup>INK4a</sup>*, *p14<sup>ARF</sup>*, *MGMT*, *GSTP1*, and *RAR $\beta$ 2*), 5-methylcytosine DNA content, and LOH were examined. In most tumors from *BRCA1* carriers, a high rate of LOH at the *BRCA1* locus was found consistent with the expectation of biallelic *BRCA1* inactivation. None of the 21 *BRCA1* familial tumors with LOH at the *BRCA1* locus had *BRCA1* methylation. Of

the two tumors that retained both alleles, one was hypermethylated at the gene promoter CpG islands. Thus, *BRCA1* promoter hypermethylation may have a role as a second event of inactivation in *BRCA1* families, but this mode of inactivation is infrequent owing to the dominance of genetic deletions as a second hit (127).

Gene methylation patterns occurring in hereditary breast cancers were found to be distinct from those of sporadic tumors (127). *BRCA1*-mutated tumors resembled sporadic breast cancers for methylation frequencies of *p16<sup>INK4a</sup>*, *CDH1*, and retinoic acid receptor (*RARβ2*) but had a much lower frequency of glutathione S-transferase P1 (*GSTP1*) methylation (24 vs 4%;  $p = 0.0001$ ). In contrast, *BRCA2*-associated tumors had a slightly higher frequency of *p16<sup>INK4a</sup>* methylation (26 vs 15%;  $p = 0.13$ ) and a lower frequencies of methylation of the other genes ( $p = 0.02 - 0.07$ ). Three *BRCA2*-mutated tumors displayed *BRCA1* aberrant methylation. Simultaneous LOH of both *BRCA1* and *BRCA2* loci happens in sporadic breast tumors, and a *de novo BRCA1* mutation has been found in a *BRCA2* mutation patient (161). The epigenetic profiles of familial breast cancers negative for *BRCA1/2* appeared to be a merger of those of *BRCA1* and *BRCA2* patients. The hereditary tumors from *BRCA1* and *BRCA2* patients displayed global hypomethylation that did not differ statistically between hereditary tumors and their sporadic counterparts ( $p > 0.05$ ). Several conclusions were drawn from this study. First, familial tumors are 'pushed' through the tumorigenic pathway owing to their initial germline mutation, but other genetic and epigenetic lesions are also necessary. Epigenetic changes in familial breast cancers are similar to sporadic forms, but with some qualitative and quantitative differences. Although overall methylation levels are comparable, the methylation of certain CpG islands differs between sporadic and inherited forms of cancer and deserve further analysis. Most notably, hypermethylation of gene promoters can frequently play a direct role in the silencing of wild-type genes as either a primary or second hit in both genetic and sporadic forms of the disease (127,159). Detailed analysis of gene methylation profiles of hereditary breast cancers may improve molecular monitoring of carriers and is a likely potential target for future therapeutic approaches (157).

## Conclusion

Morphological features of *BRCA1*-associated, *BRCA2*-associated, and sporadic breast cancers are different and may be helpful when selecting cases to test for *BRCA1/2* mutations. The data reviewed here showed that *BRCA2*-associated cancer profiles are similar but not identical to *BRCA1*-associated tumor profiles, and the data provided evidence that cancer evolution proceeds down different routes in each group, consistent with the arguments that the BRCA proteins perform distinct functions in overlapping biological processes. The unique features of tumors from germline *BRCA1* and *BRCA2* mutation carriers support a tumor progression model in which early loss of BRCA causes defects in chromosome structure, cell division, and viability, so that a *BRCA*-deficient cell must acquire additional alterations that overcome these problems and presumably force tumor evolution down a limited set of pathways. The dissection of those pathways is very important in the diagnosis, treatment, and prevention of breast cancer.

The study of familial breast cancer is in its infancy. Collaborative efforts of several research groups, using high-throughput methods such as FISH, cDNA microarrays, proteomics, CGH, and biostatistics along with IHC, are required to avoid discrepancies among studies primarily due to the low number of cases available for analysis. Further characterization of hereditary breast cancer at the molecular level will lead to improved

understanding of its pathogenesis, which will ultimately lead to improvements in diagnosis and treatment (15).

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# 5

## Cellular and Tissue Markers in Solid Tumors

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### Introduction

This chapter discusses the present and potential use of cellular and tissue markers for the management of patients with solid malignant tumors. It highlights potential developments within this area, with a focus on breast cancer, because this tumor type has served as a model for previous studies on prognostic and predictive factors.

### Preclinical Medicine and Clinical Oncology

Over several decades, we have experienced remarkable advances in the understanding of basic cellular and molecular biology. The cornerstones of this development were the discovery of oncogenes in human cancer and the identification of tumor suppressor genes (TSGs) (reviewed in ref. 1). The detailed characterization of different cyclins and cyclin-dependent kinases (CDK), responsible for the immediate regulation of various phases of the cell cycle, are other major achievements (reviewed in refs. 2 and 3). The factors involved in cell-cycle regulation may have a role as diagnostic markers and possible targets for antiproliferative drugs.

The diagnostic tools used in clinical cancer medicine have improved considerably (4). Population-based mammography screening programs for early diagnosis of breast cancer have statistically significantly improved breast cancer survival (5). The use of early adjuvant therapy of systemic micrometastases has statistically significantly improved the survival for breast and colorectal carcinoma (6–9). Despite these latter achievements, undertreatment and overtreatment should be avoided. A better use of cellular and tissue markers may help obtain these goals. The application of microarray technology may allow fingerprints of each primary breast cancer to be created, which in turn may improve prognostic information (10). Expression profiling suggests that breast cancer, as a morphologic entity, may be subdivided into five or six types with clearly dissimilar prognoses (11). In addition, array profiling can be used to separate sporadic breast cancers from breast cancers with *BRCA1* and *BRCA2* mutations (12). The present array technologies based on RNA expression profiles require RNA enrichment from fresh frozen tumors, and supplemental tumor material may be used to find better prognostic and predictive factors, with the aim of improving individualized treatment.

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Another way to diminish overtreatment may be provided by techniques that identify micrometastases. Microscopic identification of epithelial, breast cancer-like cells in the bone marrow has been done for decades (13,14). The prognostic value of micrometastases in relation to nodal status has been shown in one study, but the results need to be confirmed in prospective studies (15). Using more advanced technology, it may be possible to monitor the effect of adjuvant chemotherapy by analyzing epithelial marrow cells before and after chemotherapy, thereby using these cells for their predictive value.

Despite the achievements by use of adjuvant therapy for breast carcinoma and colorectal cancer Dukes' B and C (16,17), such tumors, together with other common solid malignant tumors, are still major killers. Conventional therapy modalities, such as surgery, radiotherapy, chemotherapy, and hormonal manipulations, have limited the effect in metastatic conditions.

### **Potential Obstacles**

The use of advanced and complicated diagnostic procedures requires more resources than are needed with the strategies now being used. This resource need may be a complicating factor for some clinicians and scientists, particularly those who are too conservative in accepting and integrating new routines for the management of patients with cancer. The late acceptance of adjuvant polychemotherapy for patients with breast cancer with poor prognosis is an example of a very cautious approach (18). The positive effects of early therapy were first described in a randomized study published in 1977 (19). Despite the fact that a number of studies were able to repeat these data (the first overview was performed in 1985) (20), it was not until the beginning of the 1990s that this therapeutic option was generally accepted in some regions and countries. Similarly, the results from the first study with trastuzumab were presented in 1998 at the meeting of the American Society for Clinical Oncology, at which Slamon et al. (21) demonstrated statistically significantly improved overall survival by adding this compound to standard chemotherapy as first-line therapy for metastatic breast cancer. Trastuzumab was available for clinical trials in Sweden in 1999 and received marketing approval in 2000. Nevertheless, the use of trastuzumab has regional differences (22). This circumspective attitude among physicians and scientists, combined with the economic strain on the health sector in many Western European countries, constitutes complicating factors to the introduction of new therapeutic molecules (18).

Another example of conservatism, skepticism, and the reluctance to accept new therapies is the slow acceptance of fine-needle cytology in the United States for the diagnostic management of patients with cancer (23). The economic factors regulating management of patients may influence the selection of method. All new principles of diagnostics, including the use of cell and tissue markers to select better therapeutic options, can be said to have met some reluctance.

### **Prognostic and Predictive Factors for Management of Cancer**

A long list of established prognostic factors based on clinical parameters exists, including TNM staging, morphologic parameters, proteolytic enzymes, mutant oncogenes, and TSGs with functional changes (Table 1). This arsenal of factors allows more accurate prognostic information for groups of patients, but not for individual patients at this time.

**Table 1**  
**Single Biomarkers with Established or Potential Prognostic and/or Treatment Predictive Information**

Endocrine	ER, PR
Proliferation	SPF, <sup>3</sup> H-thymidine labeling index, Ki-67
Oncogenes	<i>c-erbB-2/HER2-neu (EGFR2)</i> , <i>EGFR1</i> , <i>TGF-α</i>
Tumor suppressor genes	<i>p53</i> , <i>p21</i> , <i>PTEN</i>
Apoptosis	Bcl2, Bax
Angiogenesis	VEGF, bFGF, IL-8,
Proteases and inhibitors	uPA, tPA, PAI-1, MMP

bFGF, basic fibroblast growth factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor; IL-8, interleukin-8; MMP, matrix metalloproteinase; PR, progesterone receptor; SPF, S-phase fraction; TGF, transforming growth factor; tPA, tissue plasminogen activator; UPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

The use of new prognostic factors for the individual fingerprinting of each tumor has the potential to improve prognostic information for individual patients. Estrogen receptor (ER) and progesterone receptor (PR) status is a good example of a useful predictive factor for the selection of hormonal therapy in patients with breast cancer (24).

### **Morphologic Tumor Diagnosis and Diagnostic Tools**

The classic diagnoses of malignant tumors have been based primarily on the microscopic examination of representative portions of the tumor. The histopathologic description of a malignant tumor is based primarily on the visual comparison between tumor tissue and normal tissue. This method is also used for the grading of malignancies. The histopathologic classification, grading, and staging systems have been shown to be largely reproducible and very useful for the clinical management of patients with cancer. It is uncertain, however, whether this type of morphologic classification will be used in the future for all tumor types. During the last 20 yr, methods based on new understanding of molecular biology have been introduced, suggesting the possibility of measuring a single gene and its protein's alterations as a potential prognostic and/or treatment predictive indicator. Because most of these studies were retrospective, they must be confirmed in large prospective studies. To date, only prospective studies in urokinase plasminogen activator (uPA)/PAI-1 (discussed later) have been done (25). The advantage of population-based studies should not be underestimated, however. Many clinical trials have rather narrow inclusion criteria that do not reflect the typical patient population seen in the community clinic, and retrospective population-based trials would contribute important information.

### **Primary Tumors vs Metastases Biopsy and Noninvasive Techniques**

A potential shortcoming of diagnostic procedures is that almost all studies are focused on analyses of the primary tumors because the metastatic area is rarely studied morphologically. It may be important from a clinical perspective to verify that a patient has a relapsing malignant disease rather than a benign lesion or metastases from another primary malignant tumor. The biopsies used for investigations of metastatic lesions should be safe to perform, and, ideally, the method should be applicable for repeated investiga-

tions of the tumor lesions to be able to monitor dynamic changes over time in both the primary tumor and the metastases.

Biopsy techniques not requiring open surgery have developed markedly during the last few years. It is now possible to safely perform ultrasound and computerized tomography (CT)-guided biopsies in the outpatient setting to determine the extent of the primary disease and any metastases (26–35).

Rapid development will probably occur for noninvasive diagnostic procedures aimed at mirroring the effect of certain therapeutic modalities in relation to the baseline status. One such example is the positron emission tomography (PET) technique (36–41). This technique could be used to visualize different metabolic steps and processes in tumors and surrounding normal tissues. PET also could be used for dynamic studies of metabolic marker changes in tumors in relation to a given therapy, receptor occupancy, and pharmacokinetic studies. In addition, it could be used to identify potential tumor-specific markers and to increase the selectivity of different therapeutic modalities, thereby increasing the therapeutic ratio. Morphologic examination and PET methods usually do not completely reflect the tumor cell heterogeneity of most solid malignant human tumors. In the future, biopsies and noninvasive schemes must be refined to identify heterogeneity and minute changes during tumor progression.

### ***Use of Molecular Targets for Tailored Therapy***

Biologically targeted therapy is another possible use for markers, as exemplified by trastuzumab. Trastuzumab is a humanized monoclonal antibody (MAb) that is directed toward the extracellular domain of the receptor tyrosine kinase (RTK) *c-erbB-2/HER-2-neu*. The addition of trastuzumab to standard chemotherapy improves the time to progression and the overall survival in the metastatic breast cancer setting (21). Apart from trastuzumab, none of the other targeted therapies in clinical trials have been administered based on expression of the target molecule in the primary tumors or in metastatic tissues. False results may be reported in trials, because patients without the actual target tumor may be included in the analyses, thereby diluting the results into a negative study.

Very likely, the histopathologic classification and grading systems will be supplemented with additional information from genotypic and phenotypic factors. These factors may possibly be used to determine individual therapy. Phenotypic and genotypic markers must prove their worth, however, in large-scale, prospective, clinical trials.

### ***Identification of Gene and Protein Expression Profiles Using Array-Based Techniques***

In 1995, Schena et al. (42) described the first high-density cDNA microarray enabling parallel analysis of expression of a large number of genes. Later, the use of cDNA microarrays for the study of gene expression patterns in cancer was introduced (43). Specific expression profiles were found to correlate with both excellent and poor prognoses (44). New techniques can determine up to 1500 proteins in one sample. Different protein expression profiles have been found in prostate hyperplasia vs invasive cancer (45). In breast cancer, six subtypes have been described according to grouping by gene expression profiles and hierarchical clustering (11). A group in Amsterdam has shown a gene expression profile using 70 genes that is predictive of a shortened interval to distant metastasis (<5 yr) (10). This gene expression profile has been further confirmed in a cohort of 261 premenopausal women with primary breast cancer (age <55 yr) (46).

The same approach also identified a poor-prognosis group among 144 node-negative women, who had 66 and 55% relapse-free survival at 5- and 10-yr follow-up, respectively ( $p = 0.0001$ ). This finding should be compared with 96 and 83% at 5- and 10-yr follow-up, respectively, for the excellent prognosis signature provided by use of gene expression profile.

Another important step will be to identify single new genes or protein structures with currently unknown functions. In oncology, new gene and/or protein profiles with prognostic and predictive potential may soon be identified in patients with solid tumors. Ideally, the genes or proteins will be used for individualized prognostic and predictive determination, thereby allowing a truly tailored management. These profiles must be confirmed in other patient populations, and such studies are in progress.

### ***Single Prognostic and/or Therapy Predictive Factors***

During the last decades, an increasing number of potential prognostic and/or treatment predictive factors have been suggested in the literature. Some of these factors are recurring and/or constitutive factors, against which targeted therapy is under way. The advantages and disadvantages of analyses should be evaluated in light of new potential markers that will be developed. It is hoped that this technique will lead to better fingerprinting of a single tumor and improved prognosis, prediction, and therapy selections for the individual patient.

## **TP53**

### ***Background***

*TP53*, also known as *p53*, encodes a protein with a molecular weight of 53 kDa. More than 26,000 articles have been published about this TSG. The *p53* protein is encoded from a gene locus on the short arm of chromosome 17p13.1. *p53* is responsible for the control of essential cellular functions, such as apoptosis, cell-cycle control, chromosomal segregation, gene transcription, and genomic stability (reviewed in ref. 47). *p53* is a nuclear phosphoprotein consisting of 393 amino acids. *p53* gene can be activated by ultraviolet light, carcinogens, cytostatics, and radiation. The activated wild-type *p53* protein can either initiate *p53*-dependent apoptosis or, in conjunction with *p21*, inhibit the downstream CDK. *p53* can be inactivated by somatic and germline mutations or by binding to certain viral oncoproteins (e.g., human papilloma virus protein E6, SV-40 large T-antigen, hepatitis B viral X protein, adenovirus protein E1A, and oncogene MDM2).

### ***Methods for Determination of p53***

Immunohistochemistry (IHC), using different polyclonal antibodies and MAb, is the most frequently used method for *p53* determination in clinical tumor samples. The basis for IHC determination of *p53* is that the mutant protein has a markedly prolonged half-life of 4–20 h (48–51), whereas the wild-type *p53* protein has a half-life of only 15–20 min (52). The major problem for commonly used MAbs is their inability to discriminate between mutant *p53* and increased amounts of wild-type *p53* (53). The *p53* protein is usually sequestered in the nucleus, and IHC techniques provide information on the subcellular localization of the antigen (Ag) as well as its tissue distribution in malignant cells vs stroma cell and the heterogeneity within the tumor.

The degree of IHC-determined positive results has been inconsistent among studies. This discrepancy could be related to the different antibodies used for *p53* detection.



Furthermore, the choice of the fixative and the time the material is in the fixative are important (54,55). In a comparative study among the p53 antibodies Pab 1801, p53-BP-12, DO7, and CM1, Pab 1801 and DO7, after microwave Ag retrieval, gave the best localization of the p53 Ag (56). The investigators claimed that the MAb Pab 1801 was a most useful prognostic indicator (56). When the same antibody, together with Pab 240 and Signet, was investigated in another study on material from patients with colorectal carcinoma, the antibody DO7 was considered the best (57). Flow cytometry also has been used for the determination of p53 status (58).

We have compared IHC determination of p53 protein using Pab 1801 with cDNA-based sequencing of more than 300 primary breast cancer biopsies (53). With this antibody, IHC could only detect 2 of 13 deletions, none of six stop codons, and two of three insertions, but it did detect almost all point mutations, 40 of 45 (53).

We studied the luminometric immunoassay for p53 determination using Pab 1801 and DO1 antibody related to the technique previously described and cDNA-based sequencing. Again, the results were inferior to sequencing (53,59). Other studies have demonstrated that IHC produces suboptimal results when used with molecular biology-based techniques (53,60–62). In a review, Soussi and Beroud (63) showed that IHC failed to detect 11.3% of the frameshift mutations and 7.5% of the nonsense mutations when related to the p53 database.

cDNA-based sequencing of p53 on homogenized material from breast cancer tumors has been compared to genomic sequencing of the same material (64). The first scheme detected 22 of 23 mutations in the exons; one stop codon was missed (64). Three further mutations in the intron and splice regions were detected with genomic sequencing. The potential clinical relevance of these mutations is unknown. We have completed a comparative study using genomic sequencing without microdissection compared with cDNA-based sequencing on the same homogenized material from 16 breast cancer samples with known p53 mutations. All 16 mutations were detected using the cDNA-based technique, but 2 were missed with the genomic sequencing-based technique because the microdissection step was omitted (64).

Sequencing is a laborious and expensive technique, which is why other molecular biologic techniques are frequently used to screen for mutations. The most commonly used technique is the combination of polymerase chain reaction (PCR) with single-strand conformation polymorphism (SSCP). The sensitivity for the SSCP technique varies from 58 to 100% in samples with p53 mutation (65,66). To obtain optimal SSCP results, different gel temperatures and glycerol concentrations must be tested. Furthermore, this method has a higher sensitivity for smaller fragments than larger fragments. Accordingly, a negative SSCP result does not exclude the possibility of a p53 mutation.

Denaturing gradient gel electrophoresis was shown to be a useful screening method for mutations (67,68). It has the advantage of being able to detect as few as 1% mutated cells embedded in cells without mutations (69). This finding may be important for detailed studies of tumor heterogeneity, and this type of information may be particularly interesting in studies of early molecular biologic events in tumor development.

Studies of loss of heterozygosity (LOH) can be used to identify a potential difference in the expression of the paternal and maternal alleles. In our study of 26 p53 mutations, we showed that 21 tumors had LOH, 4 samples were not informative, and 1 sample retained only one allele (64).

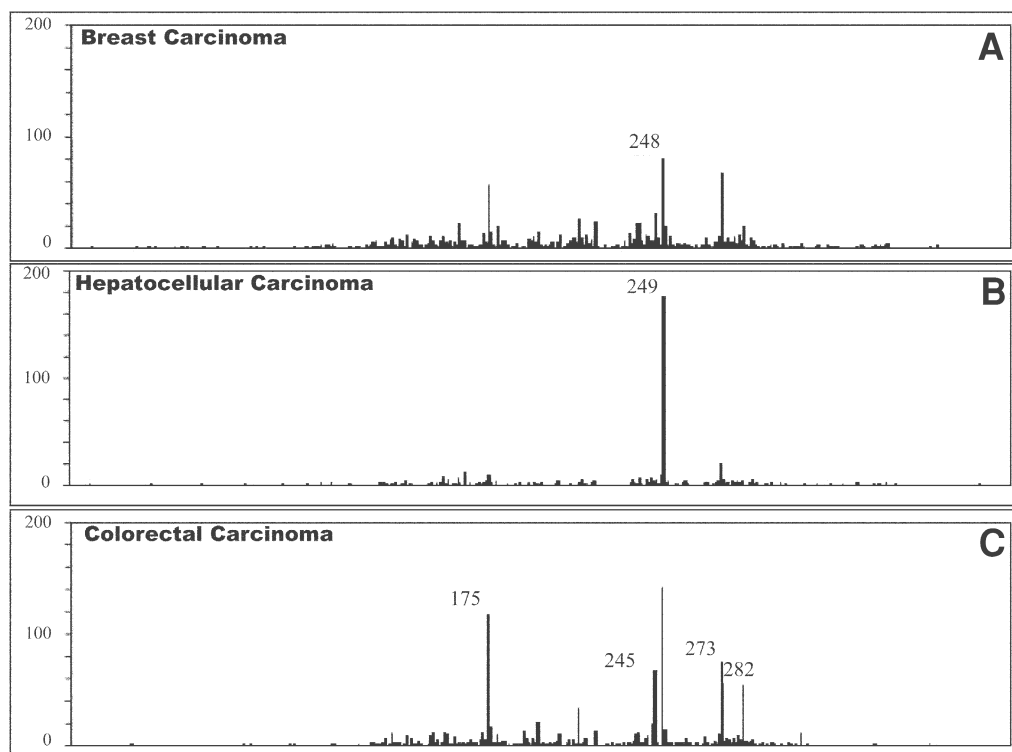


Fig. 1. *p53* mutation spectra from three cancers: (A) breast, (B) liver, and (C) colon. The y-axis represents the number of mutations reported. The x-axis represents the location of the mutations, and the numbers in the graphs indicate specific codons in the *p53* gene. The different cancers have different codons as hot spots for mutations.

### *p53* Mutations are Common

*p53* mutation has been described as the most common genetic abnormality described for human cancer. Mutations have been found in many human cancers. Fifty percent of patients with colon and lung carcinoma have *p53* mutations, but *p53* mutations are rare in patients with nephroblastomas (Wilms' tumors) or testicular teratomas (70,71). The locations of mutations within the *p53* gene vary among different malignant tumors (Fig. 1).

*p53* abnormalities can be found in dysplastic lesions of the skin, the esophagus, the bronchi, and *in situ* carcinomas of the breast [72–75]; Norberg et al., personal communication). The occurrence of *p53* mutations has been related to tumor progression, particularly in cervical, colon, and thyroid carcinoma; blast crises in chronic myeloid leukemia; and the progression of low-grade astrocytomas (76–85). We demonstrated an increased number of mutations and new mutation sites in breast cancer metastases compared to the primary lesions of some patients (86). A similar finding has been demonstrated in prostate carcinoma (87).

### *p53* and Prognoses

For many malignant tumors, patients with somatic *p53* mutations or increased *p53* protein expression have worse prognoses than patients with wild-type *p53* (54,88–105).

Patients with breast cancer and mutations in the well-conserved regions II and V, as well as mutations in the zinc finger-binding regions L2 and L3, have a particularly poor prognosis (89,92). In colorectal carcinoma, however, mutations outside the evolutionarily conserved regions are associated with a statistically significant poorer survival.

### *p53 as a Predictive Factor*

*p53* has been extensively investigated in relation to its predictive potential. Tamoxifen therapy is less effective both in the adjuvant setting and in patients with metastatic disease if the tumors contain mutant *p53* or increased *p53* protein levels (89,106). A negative *p53*/tamoxifen study with 92 patients has been published (107). The effect of chemotherapy in relation to *p53* status has been investigated in many studies without conclusive data (reviewed in ref. 108). In five of these studies, patients with increased *p53* protein levels or with mutant *p53* had a shorter relapse-free/disease-free survival and overall survival (109–113). In addition, two markedly conflicting articles have been published on the effect of anthracyclines in relation to *p53* status in patients with breast cancer. In a 50-patient study, those patients with mutant *p53* responded well to the anthracycline regimen (114); however, a study of 67 patients showed a pronounced benefit from paclitaxel, but not anthracycline, in patients with mutant *p53* (115). The reasons for these contradictory results are unknown, and the important question about *p53* status and the effect of cytostatics is being studied in a prospective, randomized study by European Organization for Research on the Treatment of Cancer (EORTC) (116). The question is: should the taxane docetaxel be given to patients with mutant *p53*, as suggested by some (109,115), or is an anthracycline regimen sufficient (114)? Furthermore, patients with mutant *p53* and acute myeloid leukemia, chronic lymphocytic leukemia, myelodysplastic syndromes, T-cell leukemia, malignant lymphoma, and ovarian carcinoma have been reported to have resistance or impaired response to conventional chemotherapy (109,117–121).

## **Tumor Proliferation**

Tumor proliferative activity influences the clinical outcome of malignant disease (122), and assessment of tumor proliferation provides predictive information regarding probability of treatment response for chemotherapy (123). Tumor proliferation may be estimated using different methodologies, which vary among institutions. Although tumor proliferation is widely used as a prognostic tumor marker, consensus on methodologic issues (e.g., the best method for determining proliferation and the criteria for interpreting the results) has not been reached. Three common methods are available for the assessment of tumor proliferation: mitotic count, S-phase fraction (SPF), and Ki-67 expression.

### **Mitotic Count**

Mitotic count is the classic method for assessment of proliferative activity in tumors, and counting the number of mitoses using light microscopy is part of some tumor-grading procedures (124). Mitotic count has been reported to be of prognostic value for several malignant diseases, including breast cancer (125); however, the method is highly observer dependent and has low reproducibility (126).

### **S-phase Fraction**

SPF is a flow cytometry method for assessing proliferative tumor activity by computerized analysis of the cellular DNA content, to identify the fraction of tumor cells in

the S-phase of the cell cycle (127). The method is usually performed on fresh or frozen tumor samples. SPF has been shown to be an independent marker for disease outcome in breast cancer (128). SPF has a low degree of observer dependency, but test results from stroma-rich tumors may be influenced by nontransformed cells. Small tumors may not have a sufficient number of tumor cells in the test sample to use SPF (129).

### **Ki-67 Expression**

The proliferation marker Ki-67 is a nuclear protein that is exclusively expressed in cycling cells. Ki-67 is expressed in all phases of the cell cycle and is, therefore, a more general marker of proliferation than SPF and mitotic count, which only consider cells in S-phase and M-phase, respectively. The function of the Ki-67 protein is undefined, but it may have several roles, including cell-cycle regulation, organization of DNA, and ribosomal RNA processing (reviewed in ref. 130). Presently, the most common method for Ki-67 determination is IHC using the MAb MIB-1. Expression of Ki-67 (using MIB-1 IHC) has been associated with poor clinical outcome in many different cancers, including breast cancer, soft-tissue sarcomas, lung cancer, astrocytomas, cervical cancer, prostate cancer, and colorectal carcinoma (reviewed in ref. 131). MIB-1 IHC requires minimal cancer tissue for determination of tumor proliferation. No general agreement has been reached on the appropriate cut off points for the measurement of Ki-67, and this issue needs clarification to improve the clinical usefulness of Ki-67 determination.

### **c-erbB-2 (EGFR2)**

The HER2/*neu* oncogene, also referred to as *c-erbB-2*, encodes for a 185-kDa transmembrane glycoprotein from the gene located at 17q21 (reviewed in ref. 132). The full-length c-erbB-2 protein comprises a cytoplasmatic domain with a tyrosine kinase activity, a transmembrane domain, and an extracellular domain that is shed from the surface of breast cancer cells (133,134). HER2/*neu* exerts its biologic activity through heterodimerization with one of the other three family members after their activation by ligand binding. Patients with anthracycline-resistant breast cancer that overexpress this receptor have shown statistically significant prolonged progression-free survival and overall survival with treatment with trastuzumab and paclitaxel (21).

### **Methods for Determination of c-erbB-2**

*c-erbB-2* status originally was determined by either Southern or Western blotting for detecting gene amplification and protein overexpression, respectively (reviewed in ref. 132). Because these methods cannot be used in the clinical routine setting, the most common way to determine *c-erbB-2* is by IHC. *c-erbB-2* gene amplification is closely related to protein expression, and if no amplification is present, the protein expression is low (i.e., not detectable by IHC) (135). For IHC testing, 17 different polyclonal antibodies and MAbs have been used (reviewed in ref. 132). Other investigators have shown a marked variability of the c-erbB-2 antibody to detect antigen overexpression in archival material (136).

Fluorescence *in situ* hybridization (FISH) quantifies *c-erbB-2* gene copies in the cancer cell nucleus, which is reportedly an accurate method in both fresh frozen and archival paraffin-embedded material (137,138). Its main disadvantages are the need for a fluorescence microscope and the loss of simultaneous histopathologic verification of the specimen (135). An unestablished alternative to FISH is chromogenic *in situ*

hybridization (CISH), in which the DNA probe is detected using an IHC-like peroxidase reaction (135). This equipment is part of routine histopathologic laboratories, and the method can be used on archival paraffin-embedded samples. CISH is faster than FISH, and verification of tissue histopathology can be done at the same time.

Presently, the most commonly used method to select patients who would possibly benefit from trastuzumab therapy is checking for IHC3+ intensity. All patients should be tested at the time of primary diagnosis.

### ***c-erbB-2 Status in Primary Tumor vs Metastasis***

The concordance between *c-erbB-2* status of the primary tumor and its metastasis is unclear. Tanner et al. (139) have shown 100% concordance for patients who developed metastasis. In another larger study, in patients with primary tumors who developed metastases 1–11 yr later, *c-erbB-2* overexpression was seen in 7 (18%) of the primary tumors vs 12 (30%) of the metastatic tumors, with a concordance of 88%. The results from the latter study point out the heterogeneity of breast cancer and the importance of obtaining the status of *c-erbB-2* or other markers at the time of therapy decision (140).

This view has been challenged by a publication claiming concordance between the examined metastases and the corresponding primary tumors using FISH and IHC (141). The study was a retrospective one that used archived metastatic samples that had been collected over many years, suggesting that patients rarely had biopsy confirmation of their metastatic lesions. Still, when IHC was used in 100 pairs of primary and distant metastatic lesions, 6 were found to have discordant *c-erbB-2* expression. Apparently, many breast cancers may develop into a biologically more aggressive disease.

### ***c-erbB-2: Correlation with Other Markers***

Increased protein expression or gene amplification of *c-erbB-2* has been shown to correlate with ER negativity (142–151). *c-erbB-2* positivity has been associated with worse prognosis, poor histopathologic and nuclear grades, and high S-phase and aneuploidy (reviewed in ref. 132). We have shown that overexpression of *c-erbB-2* is correlated with higher levels of the angiogenic factor vascular endothelial growth factor (VEGF), but with a wide spread ( $p < 0.001$ ;  $r = 0.145$ ) (152).

### ***c-erbB-2 as a Prognostic Factor***

Numerous studies have shown that overexpression or amplification of *c-erbB-2* is a negative prognostic factor for node-positive patients (153). Other studies have suggested that in node-negative patients *c-erbB-2* may be of no prognostic value (145, 154, 155). The negative results in the node-negative groups may result from fewer events being registered in a node-negative population (145, 154). The poorer prognosis in the node-positive group in one study could be explained by a decreased efficacy of CMF (cyclophosphamide, methotrexate, and 5-fluorouracil [5-FU]) therapy in node-positive patients who overexpressed *c-erbB-2* (145). It can be hypothesized that overexpression of *c-erbB-2* is seen more often with other aggressive biologic factors in node-positive patients compared with node-negative patients. This finding would explain differences regarding the prognostic value in the two subgroups. By contrast, other reports have shown that *c-erbB-2* status is a prognostic factor in patients with node-negative breast cancer (156, 157). A biologic explanation for the difference regarding the prognostic value of *c-erbB-2* between node-negative and node-positive patients is unclear and requires additional investigation.

### **c-erbB-2 and Tamoxifen**

The treatment predictive value for *c-erbB-2* in response to adjuvant endocrine therapy is contradictory. Preclinical data, in which *c-erbB-2* has been intentionally overexpressed in ER-positive cell lines, resulted in estrogen-independent growth that did not respond to therapy with tamoxifen (158). *c-erbB-2* positivity has been associated with an inferior response to endocrine therapy in patients with breast cancer, both for metastatic breast cancer and in the adjuvant setting (144,149,159–162). The GUN trial provides the most worrisome data, showing a poorer outcome for *c-erbB-2*-positive patients who received adjuvant tamoxifen compared with the same population not receiving systemic therapy (163). By contrast, no lack of efficacy of tamoxifen was seen in 205 *c-erbB-2*-positive patients with advanced breast cancer (164).

Several retrospective studies discussing *c-erbB-2* as a negative predictive factor for endocrine therapy have included patients with ER-negative disease or unknown ER status. The poor outcome seen for *c-erbB-2*-positive patients after tamoxifen treatment was postulated to reflect to some extent the negative correlation between steroid receptors and *c-erbB-2* (164). Another extensive report, not supporting a significant interaction between *c-erbB-2* and sensitivity to tamoxifen, included 8451 patients who showed a similar effect by tamoxifen irrespective of *c-erbB-2* status (165). Patients in this study received additional anthracycline-containing chemotherapy, which may have influenced the results.

The type of preoperative endocrine therapy does not affect proliferation rate in ER/*c-erbB-2*-positive tumors in contrast to ER-positive/*c-erbB-2*-negative tumors (166). Another study in the neoadjuvant setting randomized patients to treatment with an aromatase inhibitor or tamoxifen; patients receiving aromatase inhibitor had overall responses of 60 vs 41% in patients receiving tamoxifen. This difference was more pronounced in patients with tumors overexpressing *c-erbB-2* and/or epidermal growth factor receptor 1 (EGFR1) with overall response of 88% vs 21%, respectively (167). The lack of efficacy by tamoxifen can be explained by a “cross-talk” between *c-erbB-2* and ER, with a ligand-independent ER phosphorylation. Preclinical data have suggested that ER-dependent transcriptional activity in a HER-2-positive cell line can be repressed by estrogen withdrawal but not with tamoxifen. This finding supports the data from a study by Kurokawa et al. (168), in which activity by an aromatase inhibitor that decreases the estrogen levels was seen despite *c-erbB-2*/EGFR1 positivity.

### **c-erbB-2 and Cytostatics**

The ER-positive human breast carcinoma cell line MCF-7 was transfected with *c-erbB-2*, which resulted in resistance to tamoxifen and cisplatin but retained sensitivity to doxorubicin and 5-FU (158). In a clinical retrospective study, worse survival was demonstrated for adjuvant polychemotherapy with CMF therapy given to patients with breast cancer with tumors overexpressing *c-erbB-2* (145). In the National Surgical Adjuvant Breast Project B11 study, comprising 638 patients, patients with tumors that overexpressed *c-erbB-2* had a significant benefit from the addition of doxorubicin to the melphalan and 5-FU-based regimen (169). Furthermore, in a dose-intensity study, an improved relapse-free survival was seen in patients who received standard doses of 5-FU, doxorubicin, and cyclophosphamide compared with two other cohorts that received a lower dose intensity (113).

Clahsen et al. (110) however, showed a trend for improved disease-free survival ( $p = 0.17$ ) in patients with overexpression of *c-erbB-2* who received one course of

perioperative 5-FU, doxorubicin, and cyclophosphamide compared with the control group. A borderline disease-free survival benefit was demonstrated, however, for *c-erbB-2*-negative patients who received therapy compared with patients who received no adjuvant therapy ( $p = 0.05$ ) (110). Contradictory results have been presented in a study of 103 patients with metastatic breast cancer and a study with neoadjuvant chemotherapy in which the patients received epirubicin-based polychemotherapy and doxorubicin-based chemotherapy together with radiotherapy, respectively (170,171).

### **c-erbB-2 topo IIa and Anthracyclines**

*topo IIa* is the target for topo II inhibitors such as anthracyclines. The gene is located closely to *c-erbB-2*, and *topo IIa* is concomitantly amplified with *c-erbB-2* in about 40% of *c-erbB-2*-positive tumors (172). Other data have suggested that *c-erbB-2* status does not interfere in vitro with the degree of sensitivity to anthracyclines (173,174). In preclinical models, cell lines with high *c-erbB-2* and *topo II* expression were very sensitive to therapy with anthracyclines, whereas cell lines overexpressing *c-erbB-2* but not *topo II* had an intermediate sensitivity to the same treatment (174). The question of whether *topo II* rather than *c-erbB-2* contained the predictive value for response to anthracyclines was addressed retrospectively with a phase III trial in which 430 patients with node-positive breast cancer were randomized to CMF or the anthracycline-containing regimen. The results suggested *c-erbB-2* as a possible predictive marker for response to anthracycline-containing regimens, probably because of the concomitant *topo IIa* amplification seen in 38% of the *c-erbB-2*-positive patients (175). Accordingly, FEC therapy did not improve outcome in *c-erbB-2*-negative patients. Because this study included very few patients who had coamplified *c-erbB-2* and *topo IIa* ( $n = 23$ ), no conclusion can be made; however, this important question is being studied in another randomized phase III trial. That study includes 525 high-risk patients who received either an anthracycline-intensive regimen (dose-escalated and tailored FEC) or a much lower anthracycline dose followed by bone marrow-supported, high-dose therapy (176).

### **EGFR1**

EGFR1 is a 170-kDa membrane-bound glycoprotein that is expressed on the surface of epithelial cells (177). Coexpression of *c-erbB-2* and EGFR1 is known to be common in malignant tissues, including breast cancer, and this combination confers a worse prognosis. Numerous reports have indicated that EGFR1 is overexpressed in breast cancer; squamous cell carcinoma of the head and neck; glioma; and lung, gastric, pancreatic, bladder, ovarian, and prostate cancers (161,178–184). Known ligands for EGFR1 are EGF and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) (185,186), which also has a proangiogenic function. EGFR1 is a transmembrane protein that transduces a mitogenic signal on growth factor binding and receptor dimerization within the plasma membrane. Signal transduction is mediated through a domain that is homologous to the c-Src oncogenic kinase (187).

The prognostic value of EGFR1 has been investigated since 1988. A meta-analysis of 3009 patients with follow-up data and survival analyses from 16 different studies showed an inferior outcome for patients with primary breast cancers who overexpressed EGFR1 (188). In an overview consisting of 40 separate studies with a total of 5232 patients, the mean percentage of EGFR1-positive patients was 45% (range: 14–91%) (180). The most commonly used method to determine EGFR1 content has been ligand-

binding enzyme immunoassays or IHC. Compounds that block the intracellular domain of EGFR1 are in phase III trials. Despite promising data from phase II trials with ZD 1839 used as monotherapy (189), the addition of ZD 1839 to standard chemotherapy in 2130 patients with non-small cell lung cancer (NSCLC) has failed to improve time to progression or time to worsening of symptoms. It should be added that similar comparative hazard ratios for all treatment groups (between 0.9 and 1.0) were observed. No significant differences were seen with respect to overall survival, which was approximately 9 months with any of the regimens (190). Patients entering the trials were not stratified according to EGFR1 status as was done in trials with trastuzumab, and this difference may have had an effect on the outcome.

## **Tumor Angiogenesis**

The formation of blood vessels is critical during embryogenesis and regeneration of tissues after injury. Blood vessel formation is important in many disorders, including cancer. Angiogenesis is the growth of new blood vessels from preexisting capillaries and postcapillary veins. Angiogenesis is essential to the growth of both primary and metastatic tumors, because tumors need to develop an adequate blood supply (191) to grow larger than 1–2 mm (3). This tightly regulated multistep process involves degradation of basement membrane, endothelial cell proliferation, migration, tube formation, and the initiation of blood flow (192).

The ability of tumors to spread to distant sites is dependent on access to the vasculature. A higher count of microvessels and a larger surface area of these vessels are associated with an increased probability that tumor cells can enter the circulation (193). To metastasize, tumor cells need to loosen their adherence to neighboring cells, cross basement membranes, survive circulation, and establish themselves at a metastatic site. Metastatic tumor cells may remain dormant for extended periods, possibly because of a balance between proliferation and apoptosis, or because neovascularization is inhibited (194).

## **Microvessel Density**

Angiogenic activity within a tumor originally was assessed by counting vessels after staining endothelial cell markers, such as factor VIII–related antigen, CD31, or CD34. Several studies have shown that a higher microvessel density confers a worse prognosis (188,195–200). The method is somewhat subjective, and the results may not be reproducible. An alternative method is to determine the expression of proangiogenic factors quantitatively.

## **Angiogenic Factors**

A growing tumor contains a heterogeneous, highly interactive cell population, comprising malignant cells surrounded by a stromal compartment of fibroblasts, endothelial cells, neutrophils, natural killer cells, lymphocytes, mast cells, and macrophages. Tumor-associated macrophages secrete a wide variety of angiogenic factors, including interleukin-8 (IL-8), VEGF, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), TGF $\alpha$ , and TGF- $\beta$  (201,202).

The contribution of neovascularization to tumor growth lies not only in perfusing the tumor with blood-borne nutrients and oxygen, but also in the paracrine effects of endothelial cells on the tumor cells. A significant proportion of the cells in solid tumors are normal host-derived endothelial cells, and, in some cases, they represent as much



as 90% of the cell population. Endothelial cells secrete or produce a variety of cytokines and growth factors (e.g., basic fibroblast growth factor, VEGF, TNF- $\alpha$ , TGF- $\alpha$ , TGF- $\beta$ , and various ILs) that promote angiogenesis and proliferation rates (203). These factors are general stimulators of angiogenesis and activate endothelial cells, and they can act alone or in concert with other growth factors. The role of endothelial growth factors and growth inhibitors in neovascularization has been investigated in human tumor specimens. VEGF may be the predominant angiogenic factor in human breast cancer (204–208).

### **Vascular Endothelial Growth Factor**

VEGF is a potent mitogen for endothelial cells (209) and may be a crucial mediator of neovascularization in human tumors (210). VEGF exerts its activity exclusively on endothelial cells. The VEGF family consists of four structurally homologous proteins—VEGFA, VEGFB, VEGFC, and VEGFD—with affinity for three specific receptor tyrosine kinases—VEGFR1, R2, and R3. Discovered first, VEGFA was simultaneously isolated as an endothelial cell mitogen and vascular permeability factor from conditioned media from different cell lines (211,212). Later, three additional members of the VEGF family were identified and cloned (213–215). The gene encoding VEGFA is located on chromosome 6p21.3, and four isoforms named after their number of amino acids are generated through different splicing: VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>. VEGF<sub>165</sub> is the predominant isoform secreted by a variety of normal and transformed cells. To exert any biologic activity, the formation of VEGF dimers is necessary.

### **Regulation of VEGF Expression**

Some specific alteration can induce “the angiogenic switch,” including oncogenes (Ras), downregulation or mutations in TSGs (*p53*), hypoxia, inflammation, and hypoglycemia. We have shown, in a comparative study in which *p53* status was determined by two protein-based methods and one DNA-based method, that *p53* was significantly related to an increased VEGF content within the primary breast tumor. The best correlation was obtained by sequence-based data, probably because *p53* mutations produce some specifically truncated proteins (unrecognized by IHC) that had the highest VEGF levels (216). This finding suggests that increased VEGF expression is correlated with *p53* aberrations and supports the idea that angiogenesis may be regulated in part by *p53* TSG function. Our findings agree with earlier preclinical results that showed the induction of VEGF expression through the protein kinase C pathway, in the presence of *p53* mutations (217). By contrast, wild-type *p53* protein has been reported to downregulate VEGF promoter activity (218) and to upregulate expression of the antiangiogenic factor thrombospondin-1 (219).

### **VEGF as a Prognostic Factor in Primary Breast Cancer**

In 1997, Gasparini and Harris (220) first showed the independent prognostic value of VEGF in primary node-negative breast cancer. VEGF was quantitatively measured by an immunocytochemical method in cytosols from primary breast tumors, which were routinely prepared in many centers at that time for determination of ER and PR content. Seven other studies have reported similar results in both node-negative and node-positive breast cancer for recurrence-free survival, overall survival, or both (204,206–208). Note that measuring VEGF by enzyme-linked immunosorbent assay (ELISA) is not a validated method.

### **VEGF Receptors**

VEGFR1, VEGFR2 (also known as KDR), and VEGFR3 are members of the class III subfamily of RTK. All three receptors consist of an extracellular domain with seven Ig-like repeats, a transmembrane region, and kinase insert domains in their intracellular regions. The expression patterns of VEGFR1, VEGFR2, and VEGFR3 are almost exclusively restricted to proliferating endothelial cells (221); however, VEGFR2 is more widely distributed and expressed in all vessel-derived endothelial cells than VEGFR1 (222). The receptors form homodimerization or heterodimerization before phosphorylation (223). VEGFR1 has a higher affinity for VEGF than VEGFR2, although VEGFR2 is phosphorylated approx 10-fold more efficiently on ligand binding, suggesting VEGFR2 as the functionally predominant receptor for VEGF. We have found that both VEGFR1 and VEGFR2 are coexpressed with VEGF. Higher expression of both factors, but especially VEGFR2, is associated with a higher expression of *c-erbB-2* (Linderholm et al., in preparation).

### **Antiangiogenic Therapy**

Antiangiogenesis can theoretically be achieved in two ways. The first is to use specific targeted therapies, of which several are in clinical phase II and III trials in patients with metastatic colorectal cancer, breast cancer, NSCLC, and prostate cancer. These compounds consist of antibody or small peptides that are directed toward VEGF or its receptors. Results from a phase II trial in metastatic breast cancer have shown promising results, with an overall response rate of 19% with a humanized MAb directed toward soluble VEGF (224).

The second way is to use conventional cytostatics, because most of them in the preclinical setting have shown an antiangiogenic efficacy by an alternative scheduling (225). Endothelial cells recover quickly (3–4 d) after chemotherapy regardless of dose. In both a leukemia cell line and a breast cancer cell line, relatively low doses of a continuously delivered agent (cyclophosphamide) had antiangiogenic activity.

Data from the first clinical trial were recently published (226). An objective response was seen in 32% of patients treated with continuous low-dose chemotherapy consisting of 50 mg of cyclophosphamide daily together with 2.5 mg of methotrexate, twice a day, 2/wk. Two patients had complete remission and 10 patients had a partial remission, for an overall response rate of 19%. Patients remaining on therapy after 6 mo had significantly lower serum VEGF concentrations than before the start of treatment, indicating an antiangiogenic effect. An International Breast Cancer Study Group adjuvant trial is ongoing to verify the benefits of this protocol in endocrine unresponsive breast cancer.

### **Proteases: The uPA/PAI-1 System**

The ability of cancer cells to invade extracellular matrices, lymphatic vessels, and blood vessels may facilitate metastases. Proteases such as the uPA system and the matrix metalloproteinases are responsible for these processes.

uPA is by far the most extensively studied tumor proteinase. Plasminogen activation leads to formation of the broad-spectrum Ser protease plasmin that can activate the proenzyme of uPA to a proteolytically active uPA; several matrix metalloproteases are also activated (227–229). uPA and one of its inhibitors, PAI-1, are expressed at higher concentrations within several malignant tumors, including breast cancer, compared with normal nonmalignant specimens (227,229). Both uPA and PAI-1 are statis-

tically significant prognostic factors in primary breast cancer (204,230–234). Recently, an extensive, pooled analysis by the EORTC Receptor and Biomarker Group was published. With a median follow-up of 79 mo, both uPA and PAI-1 were found to be independent prognostic markers, even when conventional prognostic factors were included in the multivariate analysis (235). This finding was consistent in all patients and also when the material was analyzed in node-negative and node-positive subsets. These factors are part of a study in which the possible prognostic values have been explored in a prospective setting (25). Node-negative patients were stratified according to uPA and PAI-1 into low- and high-risk groups. Low-risk patients had low values of both factors and did not receive adjuvant chemotherapy. The high-risk group comprised patients with primary tumors with high levels of uPA and/or PAI-1; these patients were randomized to adjuvant chemotherapy with CMF for six cycles. The first interim analysis showed that the high-risk group benefited from chemotherapy: estimated probability of relapse at 3 yr was 43.8% lower than in high-risk patients who did not receive adjuvant chemotherapy. These results confirm that uPA and PAI-1 are markers of poor prognosis if patients do not receive adjuvant chemotherapy. Because patients with relatively large tumors were included (1.0–5.0 cm) and some patients did not receive therapy, the results may not be surprising. Other factors—steroid receptor negativity, histopathologic grade, or proliferation rate (besides tumor size)—did not have any influence in therapy decisions. Because these factors are used in the clinical routine to select “high-risk,” node-negative patients in many countries, evaluation of the possible additional prognostic information by uPA and PAI-1 to routine breast cancer prognostic factors would be of interest.

## Cyclooxygenase

Derived from arachidonic acid, the prostaglandins were discovered roughly 30 yr ago, and the enzymes producing these bioactive lipids were later described as cyclooxygenases (COX, prostaglandin H synthase, or prostaglandin endoperoxide synthase) (236–241). Salicylates directly target these enzymes, thereby explaining the effects of such substances on fever, pain, and inflammation (242,243).

Identified first, COX-1 was found to be a constitutively expressed, widely distributed enzyme (236,238). A second, inducible COX isoform (COX-2, cloned and characterized during the 1990s) was determined to be the main source of proinflammatory prostaglandins in pathologic conditions (237,239). In general, COX-2 is expressed only in diseased or reactive states/tissues (244).

Investigations have shown that long-term use of aspirin or other COX inhibitory compounds, called nonsteroidal anti-inflammatory drugs (NSAIDs), considerably lowered the risk of developing colorectal cancer and other epithelial neoplasias (245–247). COX-2 was found to be strongly overexpressed as both mRNA and protein in approximately 85% of the colorectal cancer samples tested in several studies (244,248,249). Soon, other epithelial neoplasias, such as cancer of the breast, urinary bladder, gallbladder, pancreas, and esophagus, were found to have varying degrees of increased COX-2 concentrations, albeit not as pronounced as in colorectal cancer (244,250–254).

## COX-2 in Breast Cancer

### Epidemiology

Published data indicate that long-term use of NSAIDs results in a small-to-moderate decrease (20–40%) in the risk of developing breast cancer (255–257). This decrease is

considerably more modest than the effect seen with colorectal cancer, which can be explained by the much lower frequency of COX-2 overexpression found in breast neoplasia (see below). Other studies suggest no such beneficial effect, but different NSAIDs were used in these studies, and the discordance could at least partly be explained by this (258,259).

### *Clinicopathology Traits, COX-2 Expression, and Prediction*

During the 1970s and 1980s, the presence of COX-2 in breast neoplasia was revealed indirectly by measuring the prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) content, which was increased according to some researchers (260,261). The first studies showing COX expression in mammary tumors used either reverse transcriptase PCR (RT-PCR) or IHC protocols, and most showed a tendency toward increased COX-2 expression (244). Although crucial, these studies were small pilot studies and were not uniform in design, drugs, or patients. In a more recent study, 1500 extensively documented tumors were studied retrospectively, and the archival tissue was arrayed to facilitate IHC high-throughput data collection (253). In this study, 40% of the samples had moderate-to-high COX-2 expression levels, and this finding was later corroborated in two other studies (262,263). In a study by Ristimäki et al. (253), COX-2 expression was more common in ductal carcinomas and correlated with aggressive parameters, such as large tumor size; high histologic grade; negative hormone receptor status; and high Ki-67, *p53*, and *Her2/neu* expression (253). The occurrence of axillary lymph node metastasis at the time of diagnosis was higher in high-COX-2 tumors (253,262). In one 40-patient study, COX-2 expression was significantly associated with several angiogenic parameters as well as with unfavorable prognosis (262,264). This finding is interesting because the angiogenic properties of COX-2 in breast tumors are quite unknown compared to our knowledge of colorectal cancer.

Aromatase is a cytochrome P450 enzyme that preferentially converts steroids into estradiol at a local tissue site, which naturally is of interest for ER-positive cancers (265). Aromatase and COX-2 expressions were strongly linked (266). In an investigation based on breast cancer cell lines, PGE<sub>2</sub> stimulated aromatase expression (267).

Univariate analyses of distant, disease-free survival, moderate-to-high Cox-2-expressing tumors showed considerably worsened prognosis (253). Multivariate analyses revealed that COX-2 is not an independent prognostic factor in high *Her2*/low hormone receptor tumors but stands alone in high hormone receptor, low *Her2/neu*, and low *p53* tumors (253). Prognosis is poor in both of these well-defined subgroups (253). Such a heterogeneous prognostic distribution may reflect different induction routes of COX-2. Indeed, *Her2* may be directly involved in breast cancer COX-2 induction (see below) (244). Of course, this hypothesis questions what is responsible for COX-2 induction in the absence of *Her2/neu* expression.

### *Procarcinogenic Actions of COX-2 in General*

Since the first reports on COX-2 expression and increased PGE<sub>2</sub> production in colorectal carcinoma surfaced, a plethora of procarcinogenic functions affecting several cornerstones of transformation have been ascribed to COX-2 (Fig. 1).

#### *Aspects Directly Linked to the Enzyme*

COX-2 is the rate-limiting enzyme for amino acid conversion into prostaglandins, since its primary product, PGH<sub>2</sub>, is the substrate for all other prostaglandins. Excess or

accumulated free nonesterified amino acids may be gated as a substrate to sphingomyelin conversion, eventually yielding highly apoptogenic ceramides (268). In this sense, the rate-limiting role for COX-2 also serves as a valve for accumulated amino acids. NSAID-induced apoptosis in colorectal cancer cells was largely due to increased free amino acid and ceramide formation as a result of COX-2 inhibition (269). On reinduction of COX-2 or another amino acid-metabolizing enzyme by means of an inducible gene construct, the apoptogenic effect of the lack of COX-2 or NSAID was reversed (269). Conceivably, free intracellular amino acids may constitute a natural mechanism for normal cell death, which is circumvented in cancer cells by increased levels of COX-2 or other amino acid-metabolizing enzymes.

### *Aspects Concerning Prostanoid Signaling*

To date, the effect of prostanoids in carcinogenesis comprises essentially four modes of promotion: cell growth stimulation, cell survival, tumor angiogenesis, and cell motility/adhesion modulation. These effects are primarily achieved through the membrane-bound G-protein-coupled PGE<sub>2</sub> receptors EP<sub>1-4</sub>, and to some extent through peroxisome proliferator-activated receptors, for which some prostanoids constitute moderate affinity ligands (244,270). Prostanoids signal proliferation in many different tumors (e.g., gallbladder, pancreatic, and colorectal cancers), which can be demonstrated both by COX-2 inhibition and by exogenous PGE<sub>2</sub> addition (254,271,272). The mitogenic response in fibroblasts and colorectal carcinoma cells elicited by EGF was markedly attenuated by NSAID pretreatment, indicating a critical role for prostanoids as cofactors to other mitogens (273–275).

Increased *Bcl-2* expression and a negative influence on nitrous oxide synthase are the most appealing explanations for the prosurvival effect seen on prostaglandin stimulation of colorectal carcinoma cell lines (270); however, in colorectal carcinoma tissue, *Bcl-2* expression is scarce, and *Bcl-X<sub>L</sub>* seems to be a major prosurvival factor because it correlates well with COX-2 expression (276,277).

Enhanced COX-2 concentrations are associated with increased adhesive, migratory, and invasive capabilities in cancer cells from liver, prostate, colon, and breast (244,270,278). The adhesive/migratory enhancement is attributable to increased hyaluronate (CD44) receptor expression and decreased E-cadherin levels, whereas upregulated metalloproteinases are thought to augment the invasive potential (279–281). These aspects were effectively abrogated by NSAID and COX-2 inhibitors.

In addition to the causal effects of COX-2 in tumorigenesis that we have reviewed, COX-2 expression in cancer cells also induces formation of VEGF, which is a well-known proangiogenic element (244,270). In membrane-separated cocultures, COX-2-expressing tumor cells caused tube formation in endothelial cells (282,283). Moreover, nonspecific and specific COX-2 inhibitors significantly impaired angiogenesis in several *in vivo* cancer models (284).

### ***Why is COX-2 Increased in Cancer?***

As reviewed earlier in this chapter, extensive laboratory investigations have revealed that COX-2 is a multifaceted participant in the procarcinogenic process. Until now, however, no mutations directly targeting the COX-2 gene or its surroundings have been identified that could explain its upregulation in cancer. At the posttranscriptional level, stabilization and destabilization of the COX-2 mRNA are considered important for COX-2 overexpression (244,270). More is known of how the increased COX-2

concentrations occur as a secondary event to the activation of oncogenes and cell-signaling pathways, although these processes also need much further investigation.

It is accepted that COX-2 responds to inflammatory factors in the microenvironment such as lipopolysaccharides, cytokines (interferon- $\gamma$ , IL-6, and TNF- $\alpha$ ), prostaglandins (possible autoregulation by PGJ<sub>2</sub>), and other arachidonic acid metabolites (possible cross talk to lipoxygenase pathways through leukotrienes B<sub>4</sub> and D<sub>4</sub>) (244,270,285). Moreover, growth factor signaling has the ability to increase COX-2 expression. Examples of deranged components are Her-2/*neu* (abnormal EGFR isoform), v-Src (mutated intracellular tyrosine kinase), k-Ras (mutated small G-protein), and c-Myc (transcriptional/cell-cycle control protein) (244,270).

Increased COX-2 expression is found in the earliest forms of spontaneous colorectal neoplasias when most of the elements are normal (270). Some evidence indicates that disruption of the tumor suppressor adenopolyposis coli (*APC*) gene, which is almost obligatory in these early lesions, also leads to upregulated COX-2. It was suggested that this could be achieved through some of the >300 genes controlled by *APC* (244,270,286), which in turn implies that several other unknown important factors may occur.

### ***Molecular Regulation and Model Systems in Breast Cancer***

Controlled by the mouse mammary tumor virus (MMTV) promoter, overexpressed COX-2 alone was enough to cause extensive breast tumor formation in mice (287). Spontaneous apoptosis appeared to decrease in normal mammary epithelium from these transgenic mice (287).

In an experimental setting using a Her-2-transformed mammary cell line, COX-2 promoter reporter assays and gel shift experiments showed that Her-2-mediated signaling regulated COX-2 transcription through cooperation between the AP-1 transcription factors (c-fos, c-jun, and ATF-2), and the PEA-3 transcription factor (288). The *in vivo* importance of this COX-2 regulation was later demonstrated in Her-2/MMTV transgenic mice, in which celecoxib (a specific COX-2 inhibitor) significantly decreased tumor occurrence (289).

### ***Methods of Detection***

Presently, no routine direct clinical method exists to determine tissue COX-2 expression. An indirect approach is to determine the PGE<sub>2</sub> content of a tissue by either high-performance liquid chromatography or ELISA methodology; although this approach provides little information on expression levels, it may be useful in certifying the presence of active and functional enzyme (260,261,285). Semiquantitative RT-PCR has been used for COX-2 expression analysis, and it would be of interest to see results from real-time quantitative RT-PCR techniques (244). Northern and Western blotting and *in situ* hybridization have been used as complementary protocols in the same studies with relatively high intermethod consistency (248,249,277).

Many commercial anti-human COX-2 antibodies are suitable for IHC. Two recent studies show that COX-2 protein expression can be assessed with a tissue array methodology, seemingly without diminishing the predictive value (253,277).

### ***Inhibition of COX-2 as Future Treatment***

COX-2-inhibiting substances have been in clinical use for many decades, and now selective inhibitors are part of our clinical reality. These pharmaceuticals make it even

more important to assess the predictive value and carcinogenic function of COX-2. A pioneering study in 1994 showed that NSAID prolonged mean survival from 250 to 510 days in patients with end-stage cancer and cachexia (290). NSAID and celecoxib significantly alleviated tumor load in patients with familial adenomatous polyposis (291,292). Some researchers have suggested that patients with tumors that express high amounts of COX-2 should be eligible for COX-2 inhibitory treatment either as part of a chemotherapy regimen or as adjuvant to surgical resection. However, this is not clear-cut because evaluations using inactive metabolites of sulindac on colorectal carcinoma cells show the same apoptosis induction as the active metabolite, and non-COX-2-expressing colorectal carcinoma cells also respond by apoptosis to NSAID treatment (270). The adverse effects of aspirin and NSAIDs are well known and may exclude them from being used as chemopreventive drugs in the future, although they may still be useful for chemotherapy (291). New selective COX-2 inhibitors seem well tolerated and potentially suited for both purposes (292). Nevertheless, further trials will have to determine the efficiency and safety in the treatment of cancer.

## Conclusion

The discovery of new genes and their corresponding proteins may become useful for the detailed fingerprinting of individual tumors. Together with a better use of the present arsenal of cellular and tissue markers, these discoveries may provide better prediction and therapy selection for the individual patient. The aim should be to tailor therapy for each patient to diminish both overtreatment and undertreatment.

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# 6

## Molecular and Pathological Characterization of Human Tumors

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Chris Jones, Ming-Qing Du, and Sunil R. Lakhani

### Introduction

Histopathologic analysis of tumors has been the linchpin of tissue diagnosis and hence classification. The information provided by histopathologists regarding tumor types and subtypes, tumor grade, and stage forms the core body of information required for clinical management. With increasing use of sophisticated radiology (ultrasound, magnetic resonance imaging [MRI]) and the implementation of screening programs for various diseases (cervical and breast cancer), pathologists are faced with problems of classifying early and borderline lesions and trying to predict their natural history. It is hoped that the new molecular techniques will help provide a molecular classification that is more robust and clinically useful.

Technological advances over the last two decades have resulted in the development of techniques such as loss of heterozygosity (LOH) (1), comparative genomic hybridization (CGH) (2), high-throughput mutational analysis, DNA sequencing, and microarrays (3). These techniques have made it easier to identify cancer-causing alterations, as well as changes in patterns of gene expression and protein function. Researchers are now in a position to investigate and quantify the many complex changes that occur during tumorigenesis. What are these techniques and how will they help in patient management?

### Immunohistochemistry

The first article depicting the use of immunohistochemistry (IHC) to stain cells was published more than 60 yr ago. For the last two decades, the technique has been used extensively in the diagnosis and subclassification of lymphomas (4) and soft-tissue sarcomas (5) and has found an increasing role in the diagnosis of cancer in other organs such as breast (6). IHC has become an invaluable tool to differentiate among the various possible primary sites in patients with metastases of unknown primary tumors (7–9). The proliferation of articles and novel molecular targets for IHC is evident from a literature search on PubMed, and a very useful site to help doctors assess the role of individual antibodies can be found at Website: <http://www.immunoquery.com>.

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IHC analysis of tumors has found a role in patient management, as illustrated by estrogen receptor (ER) and *ERBB2* in breast cancer (also known as Her2/neu). Expression is used to predict responses to tamoxifen and trastuzumab, respectively, as these therapies are designed to target these molecules (10).

## Clonality Analysis

Clonal outgrowths represent the hallmark of neoplastic proliferation and are valuable not only in research but also in diagnosis and clinical management of cancer, particularly in lymphoid malignancy. For lymphoproliferative disorders, clonality analysis is commonly performed by polymerase chain reaction (PCR)-based analysis of the rearranged antigen receptor genes, namely immunoglobulin (*Ig*) gene of B-cells and T-cell receptor (*TCR*) gene of T cells, while for nonlymphoid proliferations, clonality analysis is mainly done by PCR-based analysis of polymorphic markers of the X-chromosome.

### **Antigen Receptor Gene and Clonality Analysis of Lymphoproliferative Disorders**

The germline antigen receptor gene (*AgR*) is composed of many nonfunctional gene segments of different families including variable (V), diversity (D), joining (J), and constant (C), which must be rearranged to form a functional unit. During B-cell development in the bone marrow, the *Ig* heavy chain locus (*IgH*) first undergoes rearrangement known as VDJ recombination, followed by the *Igk* light chain or *Igλ* light chain if the rearranged *Igk* gene is nonfunctional (Fig. 1A) (11). The *IgH* recombination involves V, D, and J segments, while the *Igk* and *Igλ* recombination only involve V and J. Variable numbers of random nucleotides (N-region) are inserted at the junctions between V and D, D and J during heavy-chain rearrangement, and V and J during light-chain rearrangement. The VDJ recombination and N-region insertion create considerable sequence diversity, which forms the complementarity-determining region 3 (CDR3), the most important region of the *Ig* for antigen (Ag) binding. CDR3 is unique to each B-cell clone and therefore can be used as a clonal marker.

For clonal analysis, CDR3 is amplified by PCR using consensus primers to the framework 3 (Fr3) or framework 2 (Fr2) and the J segment. PCR with primers to the Fr3 and J segment is the preferred choice of clonal analysis because it selectively amplifies the CDR3 region and its products are small, more readily amplified from DNA samples prepared from formalin-fixed and paraffin-embedded tissues, and better resolved on polyacrylamide gels. The rearranged *IgH* gene is more commonly used for clonality analysis than the rearranged *IgL* (light chain) gene for several reasons. First, the CDR3 of *IgH* is more diversified than that of *IgL*. Second, *IgH* PCR is better established and more extensively evaluated than *IgL* PCR. Third, *IgH* PCR is applicable to all B-cell malignancies. By Fr3-J<sub>H</sub> PCR, monoclonality can be demonstrated in 70–80% of B-cell lymphomas (12). *IgL* PCR can be used in conjunction with *IgH* analysis, in particular in cases in which *IgH* PCR fails to yield a product (13). *Igk* PCR is applicable to both κ- and λ-expressing B-cell lymphomas, whereas *Igλ* PCR is applicable to only λ- but not κ-expressing B-cell lymphomas because *Igk* rearrangement precedes *Igλ* (13).

Similar to the *Ig* gene structure and recombination events in B-cells, the *TCR* loci also undergo sequential recombination during early T-cell development in the thymus.

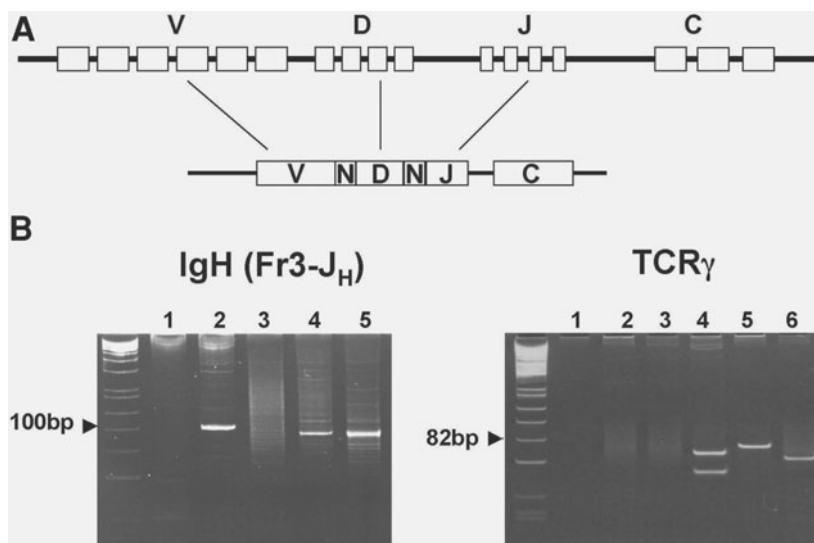


Fig. 1. (A) Recombination of various segments of germline antigen receptor gene (AgR). Variable numbers of random nucleotides (N-region) are inserted at the junctions between V and D, and D and J during VDJ recombination, forming a diversified sequence unique to each B-cell clone. V, variable; D, diversity; J, joining; C, constant. (B) (Left) Fr3-J<sub>H</sub> PCR shows a single dominant band indicative of monoclonality in a B-cell lymphoma cell line (lane 2), and a concurrent gastric (lane 4) and small intestinal (lane 5) MALT lymphoma, and a smear pattern indicative of polyclonality in a reactive tonsil (lane 3). Lane 1 is a nonlymphoid tissue sample served as negative control. (Right) TCR $\gamma$  PCR shows a polyclonal pattern in a reactive tonsil (lane 2) and a reactive lymph node (lane 3), and a monoclonal pattern in T-cell lymphoma (lanes 4–6). Lane 4 shows two strong bands indicative of biallelic rearrangements. Lane 1 is a nonlymphoid tissue sample served as negative control. (Courtesy of Dr. Tim Diss, Department of Histopathology, University College London.)

These are four TCR genes exist: *TCR $\alpha$* , *TCR $\beta$* , *TCR $\delta$* , and *TCR $\gamma$* . *TCR $\beta$*  and *TCR $\gamma$*  are the preferred targets for clonality analysis because *TCR $\alpha$*  is too complex for a simple PCR approach and *TCR $\delta$*  is frequently deleted in the mature T-cells (11). *TCR $\gamma$*  recombination involves V and J segments, while *TCR $\beta$*  recombination involves V, D, and J segments. *TCR $\gamma$*  has smaller V and J families than *TCR $\beta$* . Theoretically, the CDR3 of *TCR $\beta$*  is more diversified than that of *TCR $\gamma$* . *TCR $\gamma$*  PCR is more commonly used than *TCR $\beta$*  because *TCR $\gamma$*  rearrangement occurs in all mature T-cells, including those expressing *TCR $\alpha\beta$*  and PCR of *TCR $\gamma$*  is much simpler. *TCR $\gamma$*  PCR can demonstrate monoclonality in 70% of T-cell lymphomas (14). When *TCR $\gamma$*  PCR fails, *TCR $\beta$*  PCR may be used.

PCR-based clonal analysis of the rearranged AgR is applicable to DNA samples prepared from almost all clinical materials including both fresh and fixed tissue and cell specimens (12). It is highly sensitive, capable of detecting as few as 5% of lymphoma cells. It can be applied to a minute cell population isolated by microdissection from histologic sections or by a cell sorter. PCR products are commonly analyzed on polyacrylamide gels by electrophoresis and visualized by staining with ethidium bromide. Alternatively, PCR product can be analyzed on an automatic DNA sequencer when fluorescently labeled primers are used for PCR. Monoclonal cell populations

yield one or two dominant products, whereas polyclonal cells yield a wide range of products of different sizes that appear as a smear on gels (Fig. 1B). False-positive results occasionally occur. For example, dominant clonal B-cells have been documented in histologically apparent reactive lesions of *Helicobacter pylori*-associated gastritis (15). Therefore, results of clonality analyses must be interpreted in the context of histology.

Nonetheless, clonal analysis of the rearranged *AgR* has been valuable in several clinicopathologic applications, and its role in pathologic diagnosis is increasing, including the following areas:

- *Diagnosis*: Clonal analysis helps to define poorly understood lymphoproliferative conditions that may or may not be malignant and permits detection of neoplastic cell populations that are difficult to recognize by histologic examination and immunophenotyping. For example, clonality analysis has been instrumental in recognition of the growth and dissemination patterns of mucosa-associated lymphoid tissue (MALT) lymphoma (16–18) and enteropathy-type T-cell lymphoma (19,20), and in helping pathologists formulate criteria for histologic diagnosis of these conditions. Clonality analysis is commonly used to assess the clonal relationship of different lymphomas occurring concurrently or at different times.
- *Detection of minimal residual disease*: Because clonality analysis is sensitive, it is a very useful tool in monitoring the response of lymphoid malignancies to treatment, such as in the detection of minimal residual disease in B- and T-cell prolymphocytic leukemia and chronic lymphocytic leukemia (CLL) after chemotherapy or bone marrow transplantation (21,22). To improve detection sensitivity, clone-specific primers may be designed from the CDR3 sequence of the tumor clone and used for PCR together with consensus primers to the Fr or J region, which allows detection of tumor cells as few as 1 in 10<sup>5</sup> reactive lymphocytes (23).
- *Cellular origin and prognosis*: When primers upstream of the V segments, such as Fr1 and Fr2, are used for PCR of the rearranged *Ig* gene, sequence analysis of the rearranged V segment can reveal the cellular origin of a B-cell lymphoma. For example, B-cell lymphomas bearing germline V segment are derived from naive B cells, whereas those harboring mutated V segments are derived from germinal-center or post-germinal-center B cells (24). The former examples are mantle cell lymphoma; the latter examples are follicular and MALT lymphomas, respectively. In CLL, *Ig* gene mutation status has prognostic value: tumors with *Ig* gene mutation have a better prognosis than those without it (25).

### ***X-Linked Genes and Clonality Analysis of Nonlymphoid Malignancies***

For nonlymphoid malignancies in women, clonality assay is commonly performed by PCR-based analysis of the inactivation pattern of X-chromosome genes. The principle of the assay is based on the assumption that during female embryogenesis, each cell randomly but permanently inactivates genes on either the maternally or paternally derived X-chromosome by promoter methylation. Once methylated, the pattern of X-chromosome methylation is stably inherited by the progeny of each cell. Thus, all adult female tissues consist of a mosaic of cells with one of the two X-chromosomes methylated. Clonality assay is based on differentiation of the two differentially methylated X-chromosomes by analysis of polymorphic DNA markers (26). This analysis is commonly done by PCR-based analysis of the CAG repeat of the androgen receptor (AR) locus owing to its high heterozygosity rate. Primers are designed to flank the methylation sites and the CAG repeat. DNA samples are first digested with a methylation-sensitive restriction enzyme, allowing complete digestion of unmethylated allele



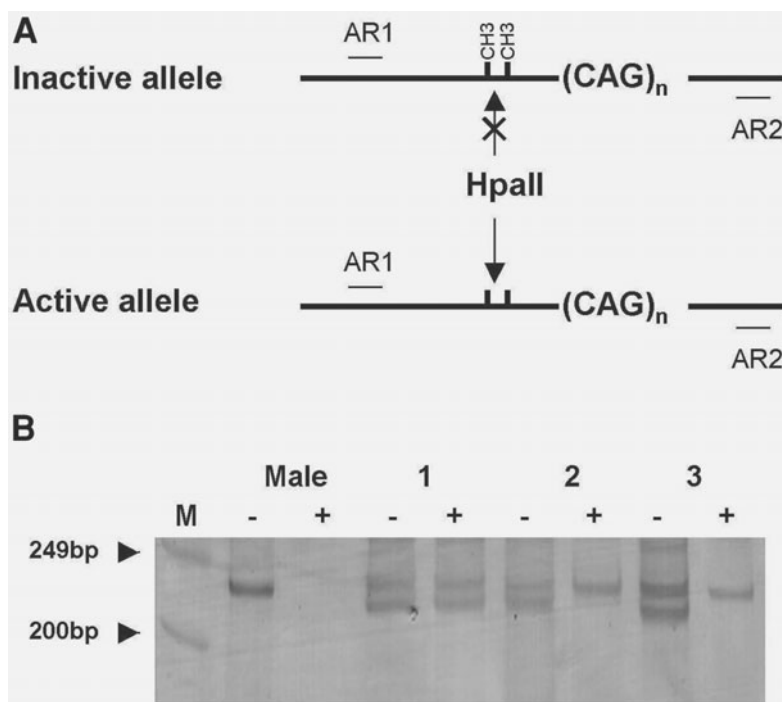


Fig. 2. (A) Digestion of DNA with methylation-sensitive restriction enzyme *HpaII* differentiates between the active and inactive allele of the X-chromosome. (CAG)<sub>n</sub> indicates microsatellite repeats. AR1 and AR2 indicate the primer position used for PCR. (B) DNA sample from a male individual (lane 1) containing only one active X-chromosome shows complete digestion with *HpaII* as PCR fails to yield any product. Case 1 shows two bands from the *HpaII*-digested sample, indicative of a polyclonal pattern, while cases 2 and 3 display only one band from the *HpaII*-digested sample, suggesting a monoclonal pattern. M, DNA weight marker; –, no *HpaII* digestion; +, with *HpaII* digestion.

but leaving the methylated allele intact as template for PCR. In a heterozygote, amplification of both alleles indicates polyclonality, whereas amplification of only one of the two alleles suggests monoclonality (Fig. 2).

A number of studies have applied clonality assays of the methylation pattern of X-linked genes to a wide range of tumors and shown monoclonality in a high proportion, demonstrating its potential value in disease diagnosis. The assay is at the research stage and is not ready for a routine clinical application since it is still controversial whether this assay can accurately define clonality because several factors may influence our interpretation of the assay (27). First, the patch size, a group of contiguous cells with common genotype, is unknown for most tissues. In a tissue composed of large monoclonal patches, a cellular expansion from multiple cells might appear to be polyclonal if it is derived from cells near the patch boundary, so that the true incidence of tumors derived from multiple cells might be underestimated. Second, X-chromosome inactivation may not always be random, and skewed inactivation pattern has been observed in some normal tissues. Paradoxically, matched normal cells are frequently not available in tumor clonality analysis.

## Molecular Cytogenetics

Most tumors have acquired clonal chromosomal abnormalities. In many instances, they are closely and sometimes specifically associated with clinically and histologically distinct tumor entity or a particular subset of a tumor entity. This association is particularly true in soft-tissue tumors, leukemia, and lymphoma. Detection of these chromosomal aberrations plays a critical role in cancer diagnosis and classification, as well as in its clinical management, including choosing treatment modality, predicting prognosis, and monitoring treatment response. Several techniques are used for detection of chromosomal abnormalities and offer tools applicable to various cancers, depending on the nature of chromosomal abnormalities and materials available for examination.

### Karyotyping

Conventional karyotyping involves culturing cells *in vitro*, preparing metaphase spreads, and staining chromosomes with giemsa, also known as G-banding. Because it requires viable cells capable of growing *in vitro*, for clinical purposes, karyotyping analysis is only widely applied to leukemias and lymphomas. As a research tool, karyotyping analysis of cancer, even in a relatively small series of cases, continues to reveal novel chromosomal aberrations because it allows a comprehensive survey of gross chromosomal aberrations including both numeric and structural abnormalities. The conventional cytogenetic analysis becomes even more powerful when it is combined with molecular genetic technology such as fluorescence *in situ* hybridization (FISH).

### Fluorescence In Situ Hybridization

FISH involves hybridization of chromosomes of metaphase spreads or isolated nuclei (interphase cytogenetics) with fluorescently labeled DNA probes (Fig. 3A). Various probes, including gene locus-specific, centromeric, telomeric, and whole-chromosome painting probes are commercially available for FISH and may be used by themselves or in combination. DNA probes can be labeled with digoxigenin and subsequently detected by the conventional immunostaining procedure, which can be viewed under light microscopy. This technique is known as chromogenic *in situ* hybridization and is applicable to formalin-fixed and paraffin-embedded sections. In general, FISH permits the detection of gene amplification, deletion, and numeric and structural chromosomal aberrations, and it is increasingly used for clinical applications. FISH is used for identification of ERBB2 (HER2)-positive breast cancers. HER2 amplification or overexpression occurs in 20–30% of breast cancers and is associated with lower overall and disease-free survival (28). Patients with HER2-positive breast cancers may benefit from therapy with trastuzumab, a humanized anti-HER2 antibody (29).

### Comparative Genomic Hybridization

For CGH, tumor and normal DNA are labeled with different fluorescent dyes (green and red) and mixed before hybridization to normal metaphase spreads (Fig. 3B). The ratio of two fluorescent colors is calculated, which allows genome-wide screening for chromosomal gains and losses. CGH can be applied to DNA samples prepared from both fresh frozen and archival fixed tissues, as well as those extracted from defined cell populations microdissected from tissue sections. CGH is currently limited for any clini-

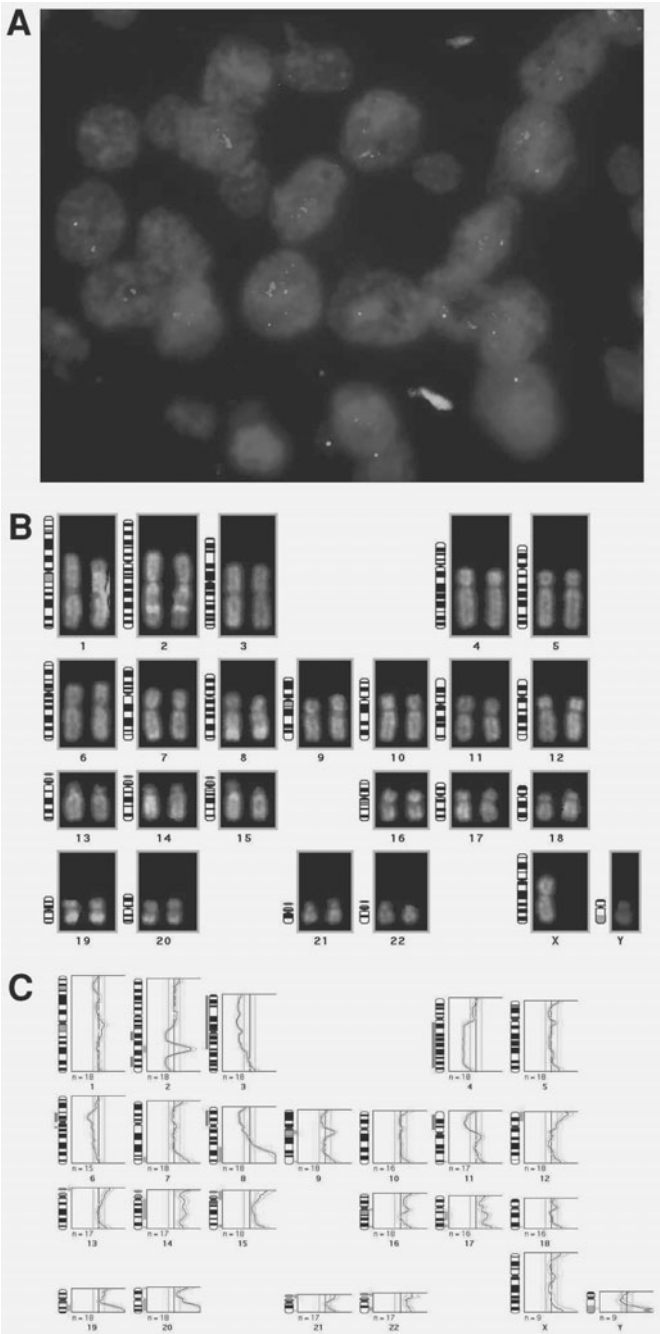


Fig. 3. (A) FISH for HER2 in breast cancer. Fluorescently labeled probe for HER2 (red) is hybridized to paraffin-embedded tissue section, counterstained with DAPI (blue). A centromeric probe for chromosome 17 (green) is included to quantify amplification. (B) CGH of invasive ductal carcinoma. The multiple fluorescence image shows regions of amplification (green) and deletion (red) in the tumor. (C) A number of metaphase spreads are analyzed, which allows calculation of mean fluorescence ratios and confidence limits across the whole genome. The figure shows that almost all chromosomes have regions of increased or decreased copy number changes. (Color illustration in insert following page 362.)

cal application, because the resolution is poor, and it is only capable of detecting high-level amplifications of approx 2 Mb, and deletions of the order of 10 Mb (30). One of the most significant contributions has been in delineating the pathways for the development of cancer from preinvasive breast disease. CGH analysis has contributed considerable data in recognizing that low-grade ductal carcinoma *in situ* (DCIS) and low-grade invasive breast cancers arise by a separate pathway from high-grade DCIS and high-grade invasive carcinoma (31,32). The application of array technology to CGH (33) has markedly improved both the resolution and throughput of the technique. The main platform developed for array-based CGH involves the spotting of BAC clones (large genomic bacterial clones—bacterial artificial chromosomes) onto glass slides (34), improving resolution to the order of 1 Mb. Microarray technology based on the spotting of cDNA clones corresponding to specific genes, developed for the profiling of gene expression at the mRNA level, may be used for genomic profiling on a gene-by-gene level by CGH (35).

### **PCR and Reverse Transcriptional PCR**

A number of chromosomal translocations can be detected by PCR. Translocations with clustered breakpoints such as t(14;18)(q32;q21), involved in the *BCL2* gene in follicular lymphoma, are readily detected by PCR, whereas those resulting in a fusion product are identified by reverse transcriptase (RT)-PCR. Both PCR and RT-PCR can be applied to formalin-fixed and paraffin-embedded tissues and defined cell populations isolated by microdissection. The methodology of PCR and RT-PCR for translocation detection is constantly undergoing improvement. Highly sensitive, real-time PCR and multiplex PCR that enables coverage of various translocation breakpoints have been developed. International collaboration is ongoing to standardize PCR protocols for the detection of chromosomal translocations (36), which will improve and increase the clinical application of these techniques. Currently, detection of chromosomal translocations by PCR and RT-PCR plays a major role in the diagnosis and clinical management of several malignant diseases.

RT-PCR is routinely used for detection of the PML-RAR $\alpha$  fusion transcript resulting from t(15;17)(q21;q22) in acute promyelocytic leukemia (APL) (21). APLs with t(15;17)(q21;q22) respond to all-*trans* retinoic acid therapy, while those lacking the translocation do not respond. In addition, monitoring the minimal residual disease by RT-PCR of the PML-RAR $\alpha$  fusion transcript is valuable in predicting prognosis and in choosing second-line therapy in patients with APL. The other newly emerged story is gastric MALT lymphoma, in which detection of t(11;18)(q21;q21) by RT-PCR of the API2-MALT1 fusion transcript identifies cases that do not respond to *H. pylori* eradication therapy (37,38). The development of gastric MALT lymphoma critically depends on *H. pylori*-mediated immune response, and eradication of the organism leads to complete regression of the tumor in 70% of cases. To determine whether a gastric MALT lymphoma responds to *H. pylori* eradication, patients need to be followed for at least 12 months. Detection of t(11;18)(q21;q21) obviates the need for prolonged follow-up with repeated gastric endoscopy and biopsy for most patients with *H. pylori* eradication nonresponsive gastric MALT lymphomas.

### **Loss of Heterozygosity**

In 1971, Knudson (39) proposed his two-hit hypothesis for the presence of cancer-causing genes that we refer to as tumor suppressor genes (TSGs). The recognition that

the second mutation that leads to inactivation of the gene is usually in the form of a large deletion led to the development of the technique of LOH in 1983 (1). The technique relies on the observation that markers (microsatellites) that are heterozygous and near the TSG would become homozygous or hemizygous in the tumor compared with normal tissue. This finding was confirmed in the case of retinoblastoma in which markers close to the gene were seen to exhibit LOH in both sporadic and familial cancers (1).

Since the introduction of the technique, numerous studies have examined LOH in many tumor types. In invasive breast cancers, LOH has been identified at almost all chromosomal locations within the genome, with high frequencies of LOH at loci on chromosomes 1p, 1q, 3p, 6q, 8p, 11q, 13q, 16q, 17p, 17q, and 22q (40). Although patterns of LOH have been reported in some series as being of prognostic significance, this information has not yet translated into routine practice.

LOH has been used to investigate precancerous lesions in the hope that patterns of LOH would help to separate lesions into benign and malignant. It has been apparent that many lesions in the breast traditionally thought of as benign are monoclonal and that considerable overlap exists in LOH between these lesions and those accepted as malignant (i.e., DCIS). Hence, no robust profiles using LOH definitively distinguish such lesions in clinical practice (31,41,42). By contrast, Barratt et al. (43) have shown that LOH profiles can distinguish subsets of colorectal carcinoma that respond to particular types of chemotherapy.

Because the LOH technique requires extraction of DNA and analysis of only a small number of microsatellites may be carried out at a time, it is labor-intensive. The information is very specific for a chromosomal location; to obtain detailed information about any region, many microsatellites have to be investigated. The use of *in situ* hybridization and modifications of these techniques have led to some progress in addressing this problem.

## DNA Methylation

The cancer cell phenotype may arise either from genetic alterations that disrupt gene function through sequence modifications (mutations) or from epigenetic events that may alter the heritable state of gene expression without changing the actual sequence of the genome. These mechanisms of altered gene expression often are found with biallelic inactivation of a given TSG occurring through combination of mutational and epigenetic events entirely consistent with Knudson's two-hit hypothesis of tumorigenesis (44). The most common form of epigenetic event in human cells is DNA methylation, the covalent addition of a methyl group to the 5' position of the cytosine ring within a CpG dinucleotide and to a lesser extent in CpNpG (45). This process is mediated by methyltransferases that are believed to function in the long-term silencing of the genome.

The current research in cancer epigenetic silencing probably has its origins in the discovery that CpG island hypermethylation was a common mechanism of inactivation of the TSG *p16INK4a* in human cancer (46–48). Since then, the list of candidate genes with putative aberrant methylation of their CpG islands has grown exponentially. Aberrant methylation of normally unmethylated 5'-CpG-rich areas has been demonstrated in, among others, the human adenomatous polyposis gene (*APC*), the breast cancer susceptibility gene (*BRCA1*), genes for tumor suppressors *p15Ink4b* and *p14Arf*, the epithelial cadherin gene (*E-cadherin*), the fragile histidine triad gene (*FHIT*), and the human MutL homolog gene (*hMLH1*) (49).

Although clinical applications of methylation perturbations in cancer have not yet been forthcoming, developments in the detection of p16 methylation in the plasma and serum of patients with liver cancer and demonstration of hypermethylation of TSG in the serum of patients with lung cancer may prove to be promising strategies toward earlier detection and hence better prevention of cancer. In addition, small molecules that reverse epigenetic inactivation are now undergoing clinical trials.

## Mutation Detection

Oncogene activation and TSG inactivation by mutation play a critical role in the genesis and progression of cancer. Detection of the cancer-associated gene mutation is expected to serve as a tool to facilitate cancer diagnosis and prognosis. Several genes such as *Ras*, *TP53* and *APC* may be potentially valuable for such clinical applications. Among these, *TP53* is the most prominent because inactivating mutation of the gene is the most common genetic alteration found in human cancers. Mutations, particularly those in the loop domain 2 and 3 (L2 and L3) of the *TP53* gene, are associated with poor survival in several cancer types such as breast, colon, non-small cell lung, and head-and-neck (50). In addition, screening for germline mutation of the cancer-associated genes such as *TP53*, *BRCA1*, *BRCA2*, *APC*, and *MSH2* can identify individuals, particularly those with familial cancer history, susceptible to cancer development and provide a basis to undertake preventive measures (51).

Mutation is commonly detected by PCR-based single-strand conformation polymorphism (SSCP) analysis or temperature gradient gel electrophoresis and confirmed by sequencing. With the development of new methods with the capacity for a high-throughput analysis, such as DNA chip analysis, it is likely that screening cancer-associated gene mutations will become a part of clinical investigations for patients with cancer.

## Expression Profiling

Analysis of changes in the profiles of gene expression (mRNA) may yield clues to the underlying molecular events in tumorigenesis. Two main platforms are available for analysis of gene expression by microarrays. The first involves the construction of high-density oligonucleotide arrays by photolithography directly onto the solid support (52). These gene chips may consist of tens of thousands of short (25-mer) probes and may be used for mutational analysis, detection of single nucleotide polymorphisms (SNP) and sequencing, and expression profiling.

The second technology used for measuring transcript levels are cDNA microarrays (53). These microarrays consist of thousands of sequences complementary to transcripts of known genes or expressed sequence tags robotically arrayed onto a glass slide. Analogous to CGH, two-color hybridization experiments may be done using RNA reverse transcribed and labeled with fluorescent dyes, such as Cy3- and Cy5-dCTP. The samples are mixed and cohybridized to the arrays in a competitive manner, and the resulting fluorescence values reveal the relative levels of each RNA transcript in the test sample compared with the reference sample. Mathematical algorithms are used by bioinformaticians to probe differences in expression patterns between sample sets (Fig. 4). These algorithms may involve the use of hierarchical clustering, principle components analysis, artificial neural networks, support vector machines, and others. Expression profiles generated by cDNA arrays can reveal similarities and differences that are not necessarily evident from traditional approaches, such as morphologic or IHC analysis.

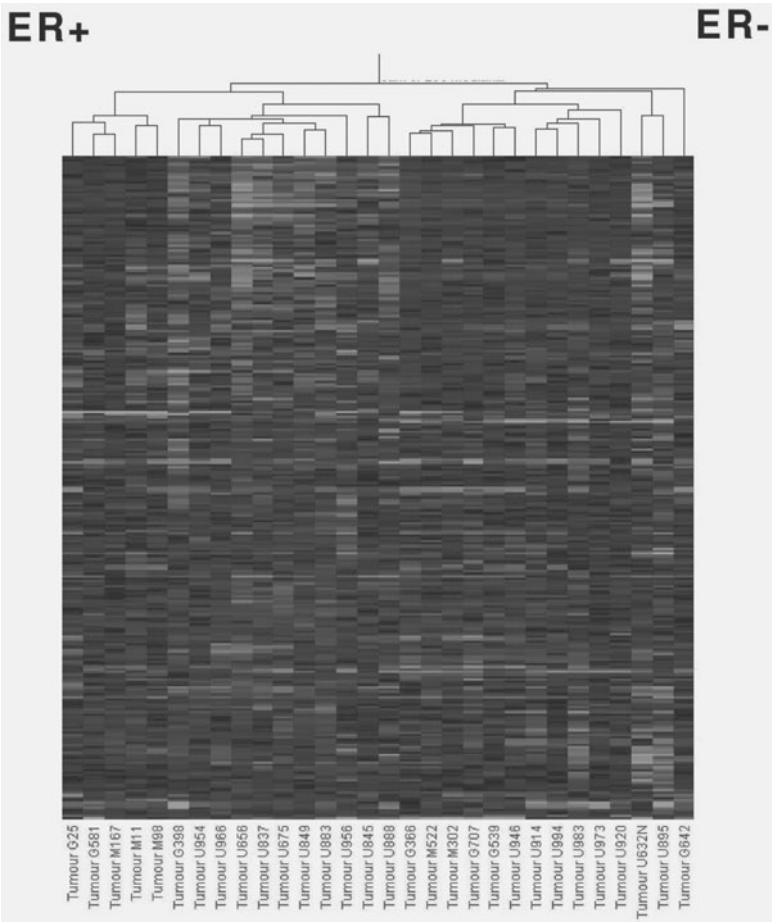


Fig. 4. Expression profiling of primary breast tumors. Hierarchical clustering algorithms can be used to subclassify samples after expression array analysis. Each row represents a gene while each column represents a sample. It is easy to appreciate that the samples are separated into the two groups of tumors representing ER+ and ER- cancers by their patterns of gene expression. ER, estrogen receptor. (Color illustration in insert following page 362.)

The power of this technique was elegantly demonstrated in several recent articles (54–56). The first demonstrated that within the morphologically homogeneous category of large B-cell lymphoma, two subtypes exist with differing patterns of gene expression (54). The second article examined expression profiles of 65 breast cancers using an array containing 8102 genes and also identified various subtypes, including an ER-negative group with basaloid features (tumors showing expression of markers seen in basal/myoepithelial cells that are a normal constituent of the duct-lobular system of the breast) (56).

These findings are not only interesting, but also reassuring. Although pathologists have been aware of basaloid breast cancer subsets, they are not currently treated as a distinct entity in clinical practice. Studies have been published to suggest that basaloid tumors have different gene expression and metastatic patterns and should therefore have a different prognosis compared with ductal carcinomas of no special type (57–59).

Array-based technologies may be used to identify specific markers that will refine this subset of breast cancers. Another article (60) further demonstrated the power of the new technology by identifying an expression signature that predicts for metastatic disease. This type of analysis will pave the way for future clinical applications.

Several important issues must be addressed before expression profiling becomes a commonplace tool in the pathologist's laboratory. Expression profiling relies on the preservation of mRNA species from the tissue of interest. Traditional formalin fixation used in routine histopathology leads to degradation of RNA, so fresh frozen material is required. Alternatively, ethanol-based fixatives may be used. Obtaining frozen material is not difficult. Freezing, however, produces artifacts by distorting the cellular architecture and cytologic features that are used to classify precancerous lesions. Laser-capture microdissection can be used to obtain defined cell populations; however, the primary diagnosis of the lesions is difficult on frozen sections. Hence, although microarray technology is producing new and valuable insights into tumor biology, we will need to await further technological development before it can be routinely used in evaluating precancerous lesions, which are now diagnosed with increasing frequency owing to improved screening programs.

A second problem in using gene expression profiles to categorize tumors is one of tissue heterogeneity. Normal tissues are composed of many cell types. Cancer cells within a tumor are heterogeneous. The use of clustering algorithms that allow *in silico* subtraction of normal tissue elements should make a significant contribution to solving the problem of contamination with normal tissue. However, this technology will not necessarily solve the problem of intratumor heterogeneity because such data are presented as a composite of the whole population, providing an average snapshot. It will be difficult to determine whether a gene that has been observed to increase expression in a tumor is upregulated in every cell of the tumor or whether its transcription is only activated in specific subclones.

Currently, the technology does not readily lend itself to formalin-fixed, archival material, and thus to microdissection strategies to address these problems. Advances in linear RNA amplification methodology are such that soon we can expect to be able to accurately profile the transcripts of small numbers of cells identified by routine histopathologic analysis, and begin to answer some questions regarding intratumoral heterogeneity, tumor–stromal interactions, and the relationship between overt cancerous cells and their benign putative precursor lesions.

An additional problem is one of data comparison among experiments and laboratories. The data derived from experiments involving oligonucleotides vs those involving cDNA are not directly comparable. Furthermore, no universally consistent standard sample can be used to normalize data. At present, little agreement exists about the number of replicates or the statistical rigor to which the data should be subjected.

## Proteomics

Protein molecules, rather than DNA or RNA, carry out most cellular functions. The direct measurement of protein levels and activity within the cell is likely to be the best determinant of overall cell function. Techniques are being developed to quantify the levels of all the proteins within a cell and compare protein levels among different cell types. Proteomic analysis, consisting of two-dimensional polyacrylamide gel electrophoresis and tandem mass spectrometry, has been used to map protein profiles in normal (61) and tumor cells (62–64). As would be expected, the studies have highlighted



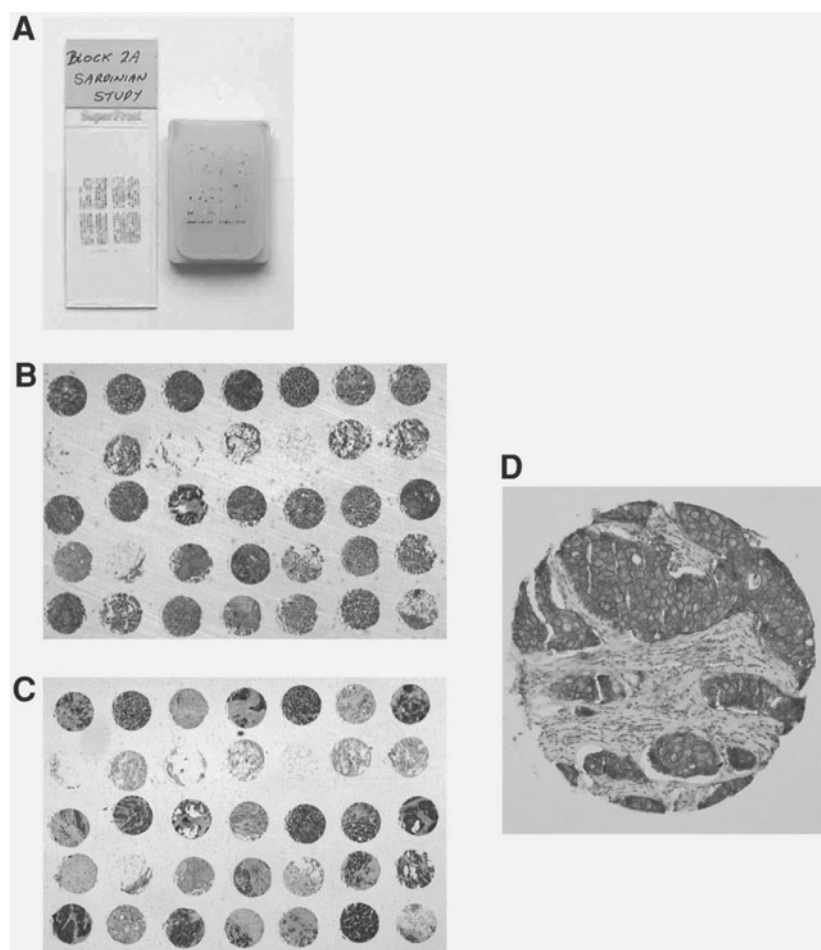


Fig. 5. (A) Low-power view of tissue array block and slide; (B) numerous 0.6-mm cores of breast ( $\times 10$  magnification) on slide stained with hematoxylin and eosin; (C) IHC for HER-2 on tissue array block; (D) high-power ( $\times 400$ ) view of one core showing positivity for HER-2. (Color illustration in insert following page 362.)

differences in protein profiles between subsets of normal cells (e.g., luminal vs myo-epithelial cells), and these will form the basis for future comparisons between the tumor and normal cells. It is unlikely that the proteome is stable over time because the cell's requirements are constantly changing as it adapts to its environment. Any proteomic data are likely to represent only a snapshot of the proteome.

At present, the technique is labor-intensive and requires large amounts of purified samples. It is not appropriate for use in clinical practice. Use of this technology will lead to the identification of new cancer-associated proteins. These proteins could then be validated on a larger number of samples using tissue arrays (Fig. 5) (65,66) and eventually developed as molecular markers for cancer diagnosis or prognosis. This development will necessitate the creation of specific antibody or *in situ* hybridization probes. At present, the technology allows identification of 2000 to 3000 gene products per experiment; this is  $<10\%$  of the cell's total proteome, and a much smaller proportion of all different isoforms of individual proteins.

Advances in technology such as nongel-based fractionation systems should allow resolution of most cellular proteins including isoforms in the future. Within the next 2 to 3 yr, a protein chip may be developed for use in clinical diagnostic practice. It would contain several hundred or thousand antibodies that could be used to measure cellular protein levels in an automated fashion.

## Conclusion

Pathologic assessment of tissues has remained the linchpin of diagnostic practice for more than 100 yr. It has become the core science of clinical medical practice, providing data for clinical management and a framework for future correlation of new markers and new therapies. In light of the new technologies, will histopathology disappear as a specialty? Some pathologists worry that one day, the surgeon will take a small biopsy of a tumor and array analysis will produce a unique fingerprint. The patient will receive individualized treatment based on the data, and the pathologist will no longer be needed.

We believe that this is an unlikely scenario, at least for the next 10–20 yr. Array-based technology will undoubtedly add to and modify current pathologic classifications. It will also modify or replace the current methods of grading tumors. Because the existing classifications are the basis for validation of the new techniques, it is more likely that the two will develop a symbiotic relationship, the new data refining the current classifications, which in turn will allow further correlation and validation of the new techniques. It is clear from preliminary molecular analysis of tumors and their preinvasive lesions that they share many genetic abnormalities. It is not easy to distinguish various types of *in situ* carcinomas from the invasive cancers. Hence, it is difficult to see at present how the array technology will help to ascertain with absolute certainty whether a sample is a pure *in situ* cancer or an invasive cancer (and hence requiring different treatments). The developments are so fast in this area that even this problem might be solved in the near future.

It is not clear whether gene expression data can be used to differentiate a primary cancer from metastases. Once microarray studies are able to answer such areas of question, they may be particularly useful in cases in which patients have metastases of unknown primary and are currently being treated on an empirical basis on a best guess. At present it is difficult to see how expression profile analysis will substitute for staging information such as tumor size, which has been shown to have good prognostic value. Even when these problems have been addressed, we may be limited by the availability of treatment options. If ductal carcinoma of the breast, B-cell lymphomas, or soft-tissue sarcomas could be separated into 20 subtypes on the basis of expression profiling but only two treatment options were available, we would not immediately realize the aim of individualized treatment. Nevertheless, this subclassification itself as well as the microarray technology will facilitate the identification of an enormous number of targets for future development of new therapeutic strategies.

Over the last decade, pathologists have grasped the nettle of quality assurance. It is recognized that pathologic diagnosis is subjective and standardization is necessary to avoid the same tumor being classified differently in different institutions with the resultant implications for therapy. Litigations for wrong diagnosis have increased. Molecular techniques, which are perceived as scientific rather than subjective, will need to undergo a continuous period of quality control. Otherwise, a cancer patient's tumor fingerprint will vary, depending on the laboratory in which the sample is analyzed.

The most realistic scenario is that in the near future researchers will use the new technologies to identify, for each tumor type, a panel of proteins that allow it to be categorized into a particular subtype. Tumors can be further characterized using IHC on tissue sections. In the longer term, the resection or biopsy sample will be sent to the pathologist, who will examine and assess the specimen by conventional means. A sample will also be used for array-based genomic, transcription, and even protein analysis. The combined data, perhaps in combination with neural networks (67), will produce a unique tumor fingerprint; however, the patient will receive one of a small number of available treatments. Over a longer time period (perhaps 10 years plus), microarray technology will result in more therapeutic options translated into the clinic.

With the current explosion of technology and data, it is important for pathologists and other clinical specialists to embrace and incorporate these changes into their training and practice. Molecular biologists will also benefit from a closer interaction with pathologists. It is, however, difficult to be dogmatic about any of these issues given the pace of progress.

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# 7

## Circulating Tumor Markers

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Alan Horwich and Gill Ross

### Introduction

The concept of a circulating tumor marker applies to a secreted chemical product of a tumor cell such that the concentration of the chemical in the blood may in some way represent a quantifiable assessment of the tumor burden at that time. The earliest example is the protein produced from myeloma cells discovered by Bence Jones in the mid-nineteenth century. Subsequently, a number of oncofetal and other proteins have proved useful and are widely available from antibody-based assays. This scene is set to expand dramatically with an increase in our knowledge of the molecular pathology of cancer subtypes and the application of genomic and proteomic analysis techniques. One example is the measurement of serum DNA concentration (1). The DNA probably derives from necrosis and apoptosis (2). The specificity can be increased by analyzing tumor DNA, such as that with allelic imbalance of sequences subject in that tumor type to frequent allelic losses (3). Alternatively, mass spectroscopy of serum proteins may provide patterns diagnostic of particular cancers. For ovarian tumors, it has been suggested that a cluster pattern can distinguish patients with cancer (4); this approach seems less successful in diagnosing early prostate cancer (5).

Currently, the range of possible tumor markers is broad, however, relatively few have been incorporated into routine oncologic practice (Table 1), possibly because any one marker is expressed in only a few tumors. This problem, however, can be addressed by a broad preliminary screen. For example, we have conducted a study of human chorionic gonadotropin (hCG), carcinoembryonic antigen (CEA), CA125, and CA19.9 in 74 patients with advanced bladder cancer and found that 43 (58%) had a significant increase of at least one serum marker (6). This information was useful because of the close correlation between clinical and marker response to chemotherapy.

The clinical roles of circulating markers might include screening, diagnosis, staging, assessment of prognosis, monitoring of response, remission, and relapse. Additionally, as relatively specific tumor products, marker substances may confer tissue specificity for immunohistochemical diagnosis and ligand-targeted techniques for imaging and therapy (7).

To be useful in clinical practice, an ideal marker should be both sensitive and specific. Furthermore, the marker test should reliably indicate the situation to which there is an appropriate therapeutic response.

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**Table 1**  
**Circulating Markers in Oncology**

Marker	Abbreviation	Tumor
Human chorionic gonadotropin	hCG	Gestational trophoblastic; germ cell, urothelial, and gastrointestinal
α-Fetoprotein	AFP	Germ cell; hepatocellular
Lactate dehydrogenase	LDH	Germ cell
Placental alkaline phosphatase	PLAP	Germ cell; lymphoma
Prostate-specific antigen	PSA	Prostate
Carcinoembryonic antigen	CEA	Gastrointestinal, especially colorectal; breast
Neuron-specific enolase	NSE	Small cell lung cancer; neuroendocrine tumor
CA125	—	Ovarian
CA19.9	—	Pancreas; gastrointestinal; ovarian

**Table 2**  
**Evaluation of Marker Test**

	Marker Positive	Marker Negative
Cancer present	A	B
Cancer absent	C	D
Sensitivity	$= \frac{A}{A + B}$	
Specificity	$= \frac{D}{C + D}$	
Predictive value	$= \frac{A}{A + C}$	

The sensitivity of a test is the probability of the test being positive in patients with the disease. Based on the symbols in Table 2, sensitivity equals  $A/(A + B)$ . The specificity of the test is the probability of a normal test result in patients without the cancer. From Table 2, specificity equals  $D/(C + D)$ . A further concept of value in judging markers is the positive predictive value, which is the probability of a patient having the cancer when the test is positive, i.e., the number of true positive results divided by the total number of positive results [i.e.,  $A/(A + B)$ ].

These relatively simple concepts become more complex for marker tests in which no clear cutoff is seen between a normal and an abnormal result, such as with a measure of prostate-specific antigen (PSA) as a diagnostic test for prostate cancer. In this setting, higher values of the marker represent a greater probability of the presence of prostate cancer and the appropriate choice of cutoff level for finding cancer may depend on patient-related factors such as the age (8).



## Use of Markers for Particular Cancers

### **Testicular Cancer**

The serum tumor markers  $\alpha$ -fetoprotein (AFP) and hCG are in widespread clinical use to aid in the diagnosis and management of patients with nonseminomatous tumors, one or both of these markers being increased in approx 75% of patients with metastatic disease (9–11). More recently, lactate dehydrogenase (LDH) has proved useful in assessment of prognosis (12). Placental-like alkaline phosphatase (PLAP) has been evaluated as a tumor marker for seminoma; however, the sensitivity and specificity of assays developed to date have not encouraged widespread use of PLAP as a serum marker (13,14).

The TRA-1-60 antigen (Ag) is expressed on embryonal cells and carcinoma *in situ*. It has been reported as sensitive, but not specific enough for clinical use (15).

#### *$\alpha$ -fetoprotein*

AFP is an embryonic protein produced by the yolk sac and subsequently by the fetal liver. It has a mol wt of 70,000 Da, is structurally similar to albumin, and probably serves a similarly diverse number of functions in the fetus. Serum concentrations decrease around the time of birth, but in adults, it has been found in the serum of patients with hepatocellular carcinoma (16,17) and subsequently in a proportion of patients with testicular nonseminoma and occasionally other tumors (18). In nonseminoma, it is usually associated with immunohistology with yolk sac differentiation. The general view is that AFP is not produced by pure seminoma despite a small number of case reports of the association.

#### *Human Chorionic Gonadotrophin*

hCG production is mainly from syncytiotrophoblastic cells. It is a hormone with a mol wt of 45,000 Da and is produced normally by the placenta. It comprises two dissimilar subunits,  $\alpha$  and  $\beta$ . The amino acid sequence of the  $\alpha$ -subunit is similar to some other human hormones, including luteinizing hormone (LH), follicle-stimulating hormone, and thyroid-stimulating hormone. The  $\beta$ -subunit is unique but shares some amino acid sequence with the LH subunit. The usual antibody-based assays for hCG are directed at the  $\beta$ -subunit but measure both intact hCG and the  $\beta$  fragments.

#### *Staging of Testicular Cancer*

One or both of the tumor markers AFP and hCG are increased in the serum of approx 75% of patients with metastatic nonseminoma. Moderate increases in hCG are found in 33–50% of patients with seminoma. In most cases, the diagnosis of a testicular germ cell tumor is not difficult on clinical grounds, although the presence of a palpably abnormal testis may indicate, as well as tumor, a possible diagnosis of local granulomatous infection, and, when painful, there may be confusion with epididymo-orchitis or torsion. The presence of an increased marker can complement further investigations such as local ultrasound. Furthermore, in approx 5% of germ cell tumors the primary site remains occult, possibly because it is extragonadal or, alternatively, because the primary tumor has remained microscopic or infarcted. In these cases, the presentation may be with lymphadenopathy, retroperitoneal or mediastinal mass, an ovarian mass; or, rarely, a pineal or pelvic tumor.

Additionally, tumor markers can help in staging assessments including assessment of prognosis. Typically, staging occurs after orchidectomy and comprises assessment of tumor markers and a computed tomography (CT) scan of the thorax and abdomen.

**Table 3**  
**International Germ Cell Cancer Collaborative Group Definitions**

Prognosis	Definition
Good (5-yr survival of 92%)	Testis/retroperitoneal primary and No nonpulmonary visceral metastases and Low serum markers AFP < 1000 ng/mL, hCG < 500 U/L, and LDH < 1.5 × NUL
Intermediate (5-yr survival of 80%)	As for good prognosis but with Intermediate serum markers AFP = 1000–10,000 ng/mL, hCG = 5000–50,000 U/L or LDH = 1.5–10 × NUL
Poor (5-yr survival of 48%)	Mediastinal primary or Nonpulmonary visceral metastases or High markers AFP > 10,000 ng/mL, or hCG = 50,000 U/L, or LDH > 10 × NUL

AFP,  $\alpha$ -fetoprotein; hCG, human chorionic gonadotropin; LDH, lactate dehydrogenase; NUL, normal upper limit.

Increased concentrations of AFP or hCG after orchidectomy do not automatically indicate the presence of metastatic disease because of the time taken for clearing these markers from the serum. The physiologic half-life of hCG determined by a standard immunoassay is approx 36 h, and for AFP, 5–7 d. Thus, especially for AFP, even patients whose tumor has been completely resected by orchidectomy may have abnormal AFP serum concentrations for some weeks, which are declining with an apparent half-life of 5–7 d. Therefore, staging assessments after orchidectomy require a sequence of markers for accurate interpretation.

AFP, hCG, and LDH are tumor products that have contributed considerably to accurate assessment of prognosis and, therefore, appropriate management of patients with metastatic nonseminoma (19). An International Germ Cell Cancer Collaborative Group prepared a database containing more than 5000 patients with advanced nonseminoma who had been treated with platinum-based chemotherapy schedules. This compilation led to publication of a consensus stratification of germ cell cancer prognosis (Table 3). Apart from a somewhat uncommon situation of primary mediastinal germ cell cancer or the presence of nonpulmonary visceral metastases (usually liver, bone, or brain), the division of patients into three prognostic groups is based entirely on marker concentrations, and these allow categorization of prognosis ranging from a group with an identified 48% 5-yr survival to a group with a 92% 5-yr survival with the presumption in this particular tumor that 5-yr survivals equate to cure rates. A similar model can apply to extragonadal tumors (20).

In this study, prognosis of patients with metastatic seminoma was dominated by the rare adverse subgroup with nonpulmonary visceral metastases. A more detailed analysis of 286 of these patients (21) and also a series from the Memorial Sloan Kettering Cancer Center (22) showed that increased serum LDH was also an independent adverse indicator as well.

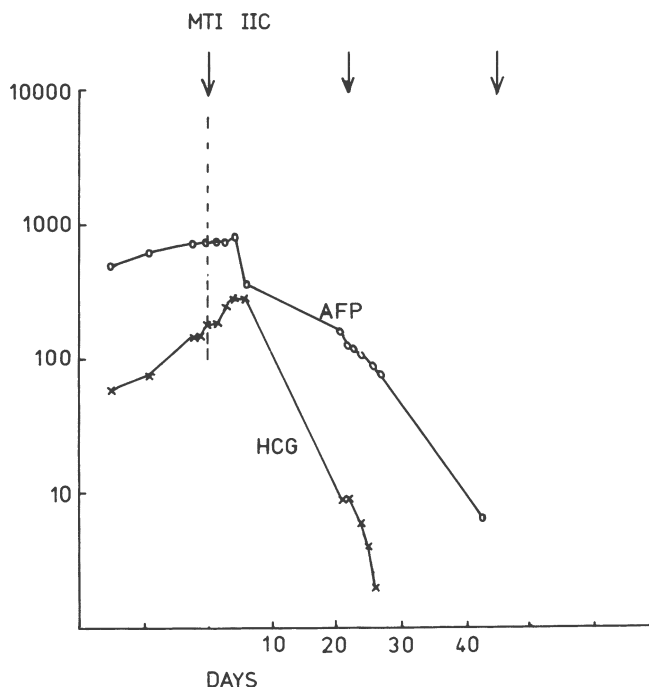


Fig. 1. Marker surge phenomenon after chemotherapy for a germ cell tumor. Arrows indicate the start of a chemotherapy cycle.

### Monitoring of Response in Testicular Cancer

Because AFP, hCG, and LDH represent tumor products, it is anticipated that a decline in the number of marker-producing tumor cells would lead to a decline in the serum concentration of the marker. It should be recognized that a change in marker concentration could follow alteration in the rate of production of marker per cell, and that the concentration of marker in the serum represents a balance between production and metabolism/excretion. Thus, although a decline in serum marker is encouraging evidence of response, occasionally the pattern of decline can be complex (23). Aspects that have been investigated include the following:

- *The surge phenomenon.* This is a transient initial increase in marker after initiation of chemotherapy (Fig. 1) that has been thought to be due either to release of stored marker or to an impact of chemotherapy on tumor differentiation (24,25).
- *The rate of serum marker decline after start of chemotherapies.* Horwich and Peckham (23) found that this was not a precise prognostic factor based on a simple comparison of marker level on d 21 of chemotherapy compared with the level before chemotherapy on d 1, with the result expressed as an apparent half-life in days. It was found that the hCG half-life in 22 patients who subsequently remained relapse free ranged from 2.5 to 9 d (mean: 4.4 d), whereas in the 7 patients who relapsed after chemotherapy, although the hCG half-life was within the same range in 6 cases, in 1 patient with very extensive disease the half-life was prolonged at 34 d, and this patient never achieved clinical or marker remission. For AFP, a narrow range of half-life was seen for patients remaining disease free (5–9 d); for 11 patients who relapsed, the range of AFP half-life was 6–14 d. Three patients with a half-life of >9 d relapsed.

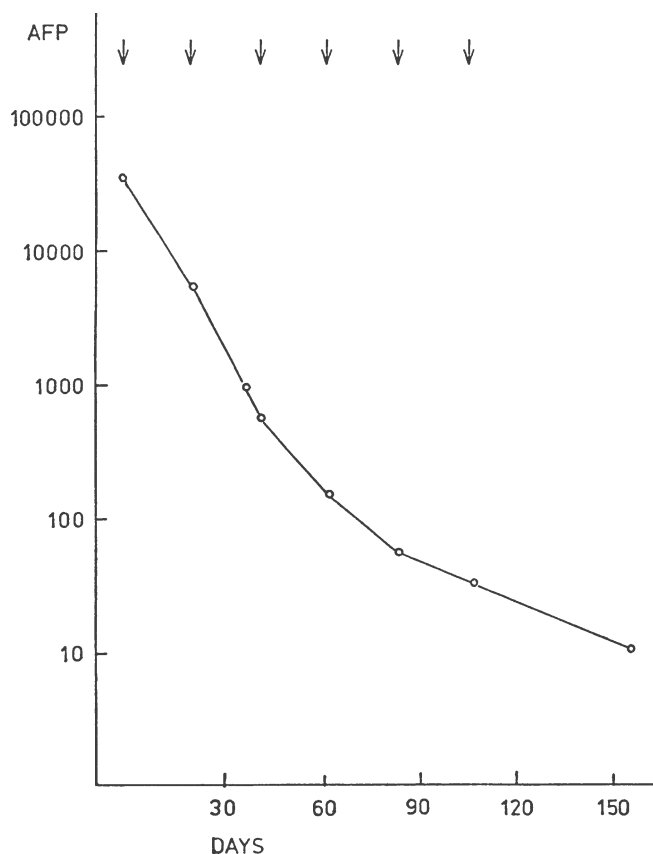


Fig. 2. Late changes in marker regression rate. Arrows indicate the start of a chemotherapy cycle.

It seems that for most patients, the initial marker pattern was determined by tumor cells that were sensitive to chemotherapy such that even those destined to relapse after chemotherapy had a dramatic initial response. The possible exception is the population of patients with drug-resistant disease and AFP-producing tumors. de Wit et al. (26) studied a group of 669 patients treated with cisplatin combination chemotherapy. Sixty-three percent had abnormal AFP at the start of chemotherapy and 58% had abnormal hCG. In the half-time analysis confined to those patients with abnormal marker concentration 3 wk after the start of chemotherapy, it was found that prolongation of either hCG or AFP half-lives did not accurately predict treatment failure. Studies at the Memorial Sloan Kettering Cancer Center, however, have identified marker regression rate as a useful predictor of outcome after chemotherapy. These studies were based on the rate of regression after two cycles of therapy with prolonged half-life defined for hCG as  $>3$  d and for AFP as  $>7$  d. Marker regression was deemed satisfactory either if less than these values or if the marker decreased to within normal limits. Satisfactory decline was associated with a median event-free survival of 20.7 mo (27).

- *Late change in marker regression slope (Fig. 2).* The significance of this pattern of response is unclear. In general, continued regression is seen as equivalent to continued response though clearly a change in slope may be a harbinger of overt marker increase and relapse. For patients presenting with high serum hCG, a slowing in the rate of decline of marker concentration in the serum is common even in patients who are cured by their initial chemotherapy (28). An isotope tracer study has suggested that this is physiologic (29).

- *Residual mass.* Just over half of patients treated with chemotherapy for bulky germ cell tumors have evidence of a residual mass at the site of their previous disease when assessed by CT scanning after completion of the course of chemotherapy. For nonseminomas, these may represent fully differentiated or mature teratoma, areas of extensive necrosis, undifferentiated persisting germ cell tumor, or a combination of these. For seminoma, the masses may be entirely fibrotic although a proportion contain residual viable seminoma. Tumor markers can have a valuable role in diagnosing the presence of persisting undifferentiated tumor in these settings and offer a useful guide to appropriate management.

### *Monitoring of Remission in Testicular Cancer*

Serum markers can help in the continued monitoring of patients after completion of their initial treatment. Across the board, 5–10% of patients who have had a satisfactory response to initial treatment will relapse. The expression of markers at the time of relapse is approximately equivalent in frequency to expression of markers at presentation. Often the pattern of marker expression changes within the individual patient and sensitive monitoring require an analysis of markers even in those whose original tumors were not apparently marker positive.

### **Tumor Markers in Prostate Cancer**

#### *Introduction*

The first marker used for prostate cancer was prostatic acid phosphatase (PAP). The acid phosphatases are found in a variety of tissues and the five isozymes have different properties and substrate specificities. PAP is predominantly composed of two of the isozymes that are also found in granulocytes and pancreas and thus may be abnormal in concentration in the serum in a range of conditions, including polycythemia rubra vera, granulocytic leukemia, Gaucher's disease, and pancreatic cancer. PAP has a mol wt of 100,000 Da and is produced by the epithelial cells lining the prostatic acini. It is found in high concentration in prostatic fluid and in the serum of >75% of patients with metastatic prostate cancer. To avoid false-positive results, it is important that a blood sample not be taken immediately after rectal examination. Currently, serum PAP measurement has a limited role in view of the relatively low sensitivity and specificity of this marker. In practice, it has been replaced by that of serum PSA.

PSA is an important marker for prostate cancer with relevance for population screening for diagnosis, for prognosis, for monitoring of treatment effects, and as a possible target mechanism in research on gene therapy. PSA is a glycoprotein with a mol wt 34,000 Da and is produced by prostatic epithelium. It is a Ser protease whose function is thought to be to liquefy seminal coagulum by proteolysis. The gene encoding PSA is on chromosome 19 and occupies approximately 6 kb.

PSA is measured in the serum by radioimmunoassay. Increased concentrations are found in both patients with benign prostatic conditions and prostate cancer. Attempts have been made to increase the sensitivity and specificity of PSA diagnosis of prostate cancer by refining the concentration using parameters such as PSA density (relating to size of the prostate gland) and PSA velocity (rate of change with time), PSA relating to age, PSA fractionation (free vs bound), and measurement of cells in the circulation expressing PSA messenger RNA.

#### *PSA in Diagnosis*

As with PAP, PSA can be increased by prior digital rectal examination although it is rare for this to cause the value to become out of the normal range (30). The incidence of

higher PSA concentrations increases with the stage of the tumor, from approx 40% in men with occult presentations, to 70% in men with tumors penetrating the capsule, and to almost 100% in men with extension of the primary tumor to seminal vesicles or involvement of local lymph nodes (31,32).

A number of large studies have evaluated PSA in the screening of prostate cancer. For example, in a study of more than 1200 men over 50 yr of age, serum PSA was found to be increased in 187 (15%), of whom 32 had cancer detected by biopsy (detection rate of 2.6%) (33). In a similar study based on 1653 patients, PSA was measured between 4 and 9.9 in 107 (6.5%) and >10 ng/mL in 30 (1.8%). Cancer was eventually diagnosed in 22% of the former but in 66% of the latter, with an overall detection rate of cancer in the study of 2.2% (34). Not all screen-detected cancers are associated with an abnormal PSA, and in typical series 20% of such tumors are associated with a normal value (35,36).

Although PSA offers a relatively inexpensive and highly acceptable screening test for prostate cancer, the rationale of this technology must be based on its specificity as well as on demonstration that early treatment of the disease improves the prognosis. A formal screening trial in prostate cancer has not been completed, and, therefore, PSA screening has not been adopted in all countries.

### *PSA and Staging*

The incidence of an abnormal PSA increases with advancing stage of the cancer and mean value increases with advancing stage, probably as a consequence of the relationship between serum PSA and the volume of the prostate tumor (37). In one study (38), the mean serum PSA was 5.6 ng/mL in men with organ-confined cancers, 7.7 ng/mL in men with localized cancers but capsular penetration, 23.2 ng/mL in men with seminal vesicle involvement, and 26.2 ng/mL in men with involved lymph nodes. The amount of PSA may be useful in the prediction of bone scan findings. In 521 men with newly diagnosed prostate cancer, of those with a PSA  $\leq$  20 ng/mL ( $n = 306$ ), only 1 man had a positive bone scan and none with a PSA <10 ng/mL had a positive bone scan (39).

### **PSA as Marker of Response to Treatment**

#### *Radical Prostatectomy*

A decrease in PSA to undetectable levels after radical prostatectomy defines a subset of patients with a better prognosis (40). This finding has been confirmed by biopsy studies after prostatectomy that are more frequently positive in men with a high PSA (41). This assay can provide a key indication for postprostatectomy irradiation. In this setting, the rate of rise of PSA can be helpful in distinguishing patients with locoregional from patients with advanced metastatic disease, since the latter tend to have a doubling time of <6 mo.

After radiotherapy for localized prostate cancer, the serum PSA has been found to decrease with a half-life of between 1 and 3 mo (42,43). This slow regression reflects the known slow disappearance of malignancy after radiotherapy, thought to be a consequence of tumor cell death occurring only on attempted cell division. A longitudinal study has suggested that PSA concentrations regress very predominantly in the first year after treatment, and continued regression after 1 yr occurred in only 8% of men (37). In another study based on 143 patients followed for a median of 27 mo after radiotherapy, 94% of those whose PSA normalized within 6 mo remained relapse free compared with only 8% of those whose serum PSA remained increased after 6 mos.

### *PSA After Hormonal Therapy*

PSA response and clinical response to hormone therapy are clearly connected (31). The degree of decrease in PSA is an indicator of remission duration (44). An increase in serum PSA after hormone therapy is a predictor of clinical progression with a mean lead time of approx 7 mo. Although the value of this finding is not well established, in prostate cancer a relationship between decrease in serum PSA and patient benefit has been seen (45–47).

## **Gastrointestinal Tumors**

### *Carcinoembryonic Antigen*

CEA is a 200,000-Da glycoprotein that was isolated in 1965 using an antibody raised by injection of an extract derived from human colonic carcinoma into rabbits. Immunochemical electron microscopy techniques can demonstrate the presence of the protein in normal colonic columnar cells.

Assays are available using both polyclonal and monoclonal antibodies. Serum CEA concentrations are increased in carcinomas of the gastrointestinal tract (GI), but can be increased in a variety of nonmalignant conditions, reducing the specificity; these include GI inflammation, collagen disorders, infection, trauma, infarction, renal impairment, and smoking. Generally, however, concentrations achieved in these conditions do not reach those documented in colonic malignancy. The low sensitivity and specificity of serum CEA precludes its routine use in screening general populations for colorectal cancer; hence, interest has turned to evaluating its use in assessing prognosis or monitoring therapy in established disease.

The value of preoperative CEA concentration as an independent prognostic marker is not clear, although amounts broadly reflect tumor burden, and increase and decrease with response to therapy (48). Serum CEA concentrations should decrease to normal within 6 wk of complete tumor resection. Fewer than 5% of patients with Dukes A colorectal carcinoma will have increased serum CEA, rising to 25% of Dukes B cases, 44% of Dukes C, and 65% of patients with metastatic disease. A number of investigators have reported that increased amounts of CEA predict an increased risk of recurrence (49–51), but others have reported its prognostic value to be limited (52,53). In a series of 377 patients with advanced colorectal cancer, serum CEA was an independent predictor of survival (54). Monitoring for recurrent disease status by serum CEA is of limited value (55). Up to 30% of cases of recurrences will have normal concentrations.

Increased amounts of serum CEA can be found in patients with advanced non-colorectal tumors, including breast, lung, cervix, endometrium, and ovarian cancer, and may be useful in monitoring response to therapy.

### **CA19.9**

CA19.9 was derived from a human colonic adenocarcinoma cell line, and several commercial kits are available for clinical measurement. Concentrations of this Ag are increased in up to 75% of patients with advanced colorectal malignancy, but its main value lies in its greater sensitivity than CEA in monitoring gastric, pancreatic, and biliary tumors (56). Recent research suggests that serial analysis of levels of CA19.9 can be used to predict response to radiotherapy in inoperable cases of pancreatic cancer (57), in which conventional imaging may have limited clinical sensitivity.

### **Ovarian Cancer and CA125**

CA125 was first reported in 1983 after a murine monoclonal antibody (MAb) was raised to a human ovarian cystadenomacarcinoma. CA125 is produced by tissues derived from coelomic epithelium, which includes the peritoneum, fallopian tube, endometrium, endocervix, pleura, and pericardium, but not the normal ovary. It is present as a cell-surface glycoprotein in approx 80% of epithelial ovarian tumors, with a serum half-life of 4 d. While concentrations  $>35$  IU/mL are seen during the first trimester of pregnancy, in a range of benign conditions (cirrhosis, endometriosis), and other advanced intraabdominal malignancies, 99% of normal blood bank donors will have concentrations lower than this. Increases  $>35$  IU/mL are seen preoperatively in  $>90\%$  of women with stage III or IV ovarian carcinoma, but only 50% of those with stage I disease. Unfortunately, the low specificity of CA125 precludes its use in screening general populations, and its sensitivity is too low to use alone in the screening of high-risk women (58). Combined with ultrasound and knowledge of menopausal status, CA125 levels provided 85% sensitivity and 98% specificity for the diagnosis of pelvic malignancy in a cohort of 143 women investigated for a pelvic mass (59,60).

Serial CA125 concentrations are the best method of monitoring response to therapy. A decrease of  $>50\%$  maintained for  $>28$  d is highly predictive of response, and, conversely, a serial increase of 50% indicates progression. In a detailed study (61), CA125 was measured during early chemotherapy in 121 women with FIGO stage III or IV ovarian cancer to investigate whether the Ag could be used as a prognostic parameter. CA125 was determined before the start of chemotherapy and 1 mo after the first, second, and third course. The Ag concentration before the start of chemotherapy held no prognostic information. CA125 was a significant prognostic parameter in all three courses but its correlation with survival improved with the number of courses. Women with marker levels ( $>100$  U/mL) 1 mo after the third course had a median survival of 7 mo, compared with a 50% 5-yr survival in women who had  $\leq 10$  U/mL and a median survival of 22 months among women with intermediate CA125 levels. Cox regression analysis of the covariation among survival, CA125, and five variables (age, FIGO stage, histopathology, tumor grade, and bulk of residual tumor) showed that the CA125 value was the most significant prognostic parameter. As a consequence of this study, the investigators suggested that chemotherapy of women with high CA125 levels 1 mo after the third course could be discontinued and replaced by palliative therapy if other curative regimens were not available. Similar conclusions were reached in a large study involving 573 cases, confirming that serum CA125 levels after three cycles of chemotherapy are predictive for the probability of achieving complete remission.

### **Breast Cancer–Related Markers**

A number of MAbs have been raised to mucins, high-molecular-weight glycoproteins produced by epithelial cells of the breast. The most heavily investigated mucin marker is CA15.3, which is increased in approx 11% of women with operable breast cancer and 60% of cases of metastatic disease. It is also increased in some 10% of women with benign breast disease. The lack of specificity and low sensitivity preclude the use of CA15.3 in screening or diagnosis of symptomatic breast disease, but serial estimations may be of value in monitoring response of metastatic disease. A prospective study was undertaken to define the optimal combination of bone scan and tumor marker assays in staging a breast cancer cohort of 157 consecutive cases.



The results suggest that in asymptomatic patients, a CA15.3 concentration <25 U/mL (upper normal value chosen as the threshold) is strongly predictive of a negative bone scan; by contrast, high tumor marker concentrations are predictive of neoplastic bone involvement. When a doubtful bone scan is obtained in a patient with breast cancer, a normal marker level makes it highly probable that bone scan abnormalities are not related to malignancy (62).

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# 8

## Proteomics-Based Approaches

### *New Opportunities in Cancer Research*

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**Rachel A. Craven, Peter J. Selby, and Rosamonde E. Banks**

#### Introduction

Understanding the molecular, cellular, and tissue changes that occur during tumorigenesis is central to the cancer research effort. The translational aspects of this field—the development of clinical applications from the laboratory findings—are aimed at improving diagnosis, monitoring, and treatment of disease, targets that are facilitated by an appreciation of the mechanism underlying pathogenesis. Approaches used to achieve these goals can be divided into two broad classes. The first of these encompasses strategies designed to identify disease markers that can be used to develop screening tools with sufficient sensitivity and specificity to detect cancer in the general population, aid in clinical cancer diagnosis, predict prognosis, and identify patients with recurrent or metastatic disease. Second are studies aimed at improving therapy, by identifying either molecules and pathways that can be exploited as targets for disease intervention or molecules that predict response or resistance to therapy with the aim of patient stratification and individualization of treatment.

Studies examining changes at the genetic level that accompany cancer progression have identified a number of genes (both oncogenes and tumor suppressor genes [TSG]) that are central to tumorigenesis. These include genetic changes underlying particular cancers that influence susceptibility, such as mutations in *BRCA1/2* (breast and ovarian cancers), *VHL* (hemangioblastomas, renal cell carcinoma, pheochromocytoma), and *HNPCC* (colorectal cancer), which are used for assessing familial risk and screening. In hematologic cancers, including leukemias, lymphomas, and myelomas, tests based on cytogenetic abnormalities are now central to disease management at many points in patients' pathway of care; however, this is not the case for most solid tumors, where most tumor markers used for diagnosis, prognosis, or monitoring are protein based. Many markers have arisen from global profiling studies or antibody generation, a prominent example being CA125. Other markers/targets result from rational design and knowledge of the underlying tumor biology such as estrogen receptor (ER) and Her2-neu treatment in breast cancer, and tyrosine kinase inhibitors (imatinib and related compounds) in leukemia and sarcoma. The requirement for a new generation of markers/targets in clinical practice to supplement those in current use is pressing,

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and increasingly it is being realized that panels of markers or targets may be required for unambiguous clinical utility, especially when considering the degrees of tumor and patient heterogeneity that are routinely encountered.

The search for new markers/targets has prompted numerous studies examining gene expression in tumors and normal tissues. Analysis at the mRNA level has increased significantly with the introduction of polymerase chain reaction (PCR)-based strategies for comparing mRNA populations and microarrays based on cDNAs or oligonucleotides that allow thousands of gene products to be analyzed in a single experiment. The data from such approaches, particularly those generated from application of microarrays, have been used to generate expression patterns for molecular classification of tumors and prediction of disease outcome (1,2).

It is becoming clear that nucleic acid-based approaches should be complemented by investigations into the functional output of the genome, the proteome, which is the set of proteins expressed in a particular cell type. The proteome is a dynamic entity that is determined by genetic events but is also affected by epigenetic, cellular, and environmental factors (Fig. 1). Studies at the protein level overcome the lack of correlation between mRNA and protein concentrations that results from control mechanisms operating at the posttranscriptional level. In addition, proteomics allows access to the further level of complexity manifested by a large number of potential posttranslational modifications, including glycosylation and phosphorylation. Furthermore, biologic fluids, such as serum, urine, and ascites, are ideally suited to analysis by proteomic-based techniques with the aim of detecting proteins shed or secreted from tumor cells.

Technological advances in protein separation, mass spectrometric analysis, and bioinformatics over the last few years have resulted in the feasible study of global gene expression at the protein level. Such studies include determining the proteome of a cell type/sample of interest, mapping the role of proteins in cellular pathways, and understanding protein-protein interactions and also include knowledge of the role of subcellular localization. Identifying protein molecules that are expressed differentially between normal and diseased states by comparative analysis is now a realistic possibility. Although it is too early to see many results from such studies translated into clinical use, the approaches hold much promise for future cancer management. In this chapter, we focus on a number of protein-based approaches that are currently being applied to the study of cancer, including several novel strategies that will clearly be instrumental in future research.

### **The Conventional Approach: Two-Dimensional Polyacrylamide Gel Electrophoresis**

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is the central separation tool in protein-profiling studies (3). In the first dimension, proteins are separated on the basis of charge ( $pI$ ) by isoelectric focusing, generally using an immobilized pH gradient. In the second dimension, sodium dodecyl sulfate-PAGE (SDS-PAGE), proteins are separated on the basis of their gel mobility, which is determined largely by their molecular weight. Protein detection is usually achieved by subsequent silver or Coomassie Blue staining or by the use of fluorescent dyes, such as SYPRO Ruby (4). Improved sensitivity can be achieved by the use of metabolic labeling and although this requires a short time in culture, it does overcome the problem of serum contamination that can result from the study of whole-tissue extracts. For comparative

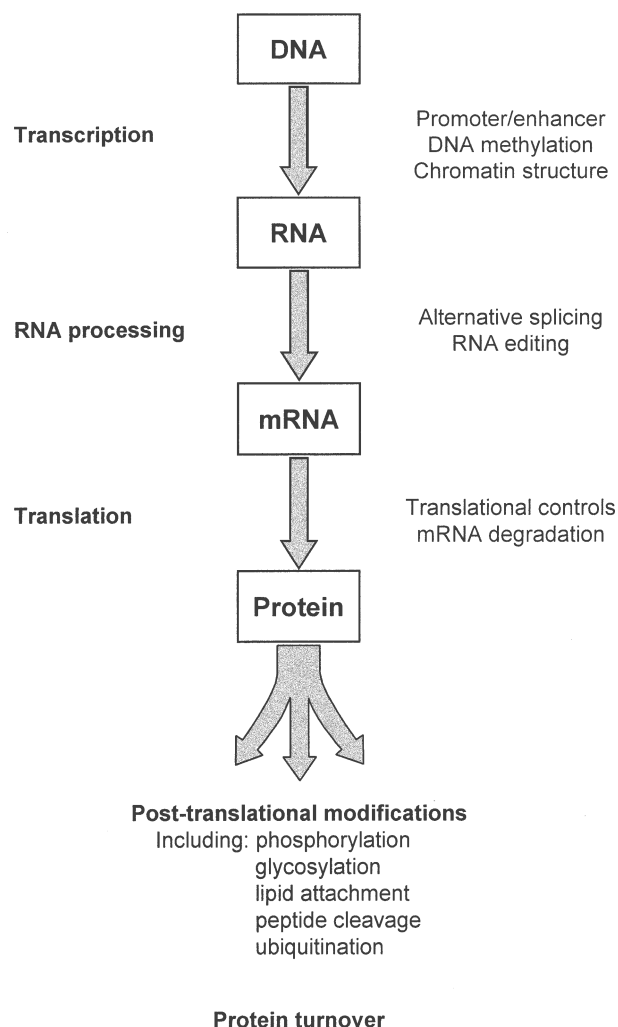


Fig. 1. Regulation of protein expression. Protein levels can be affected by regulation at several key stages by both genetic and epigenetic mechanisms. These include steps in the protein synthesis pathway (transcription, mRNA processing, and translation) as well as at the level of the mature protein (posttranslational modification[s] and protein turnover).

analyses, gels of each sample, generally run in triplicate, are analyzed either visually or using specialized image analysis software, to allow selection of potentially interesting (generally differentially expressed) protein species.

For identification of proteins of interest, spots are generally excised from the gel, digested with trypsin, and the digest products are analyzed by mass spectrometry. The masses of the peptides produced, or peptide-mass fingerprint, are specific for a particular protein, and identification can most easily be achieved by searching these masses against a theoretical tryptic digest of the proteins in a database. Alternatively, tandem mass spectrometry strategies can be used to generate fragmentation profiles of particular peptides, providing protein-sequence information.

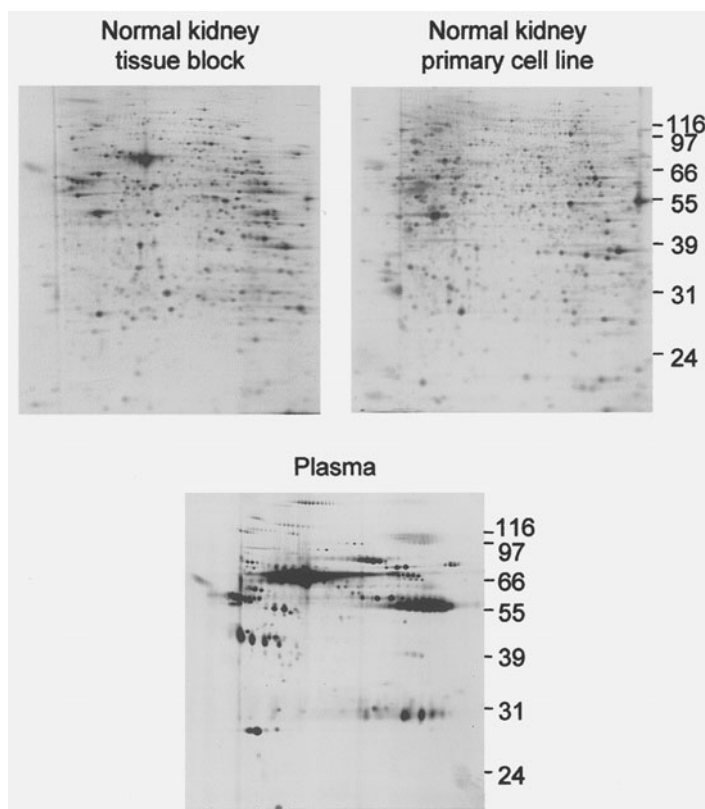


Fig. 2. 2D gels of tissues, cell lines, and fluids. Representative 2D-PAGE separations of extracts of normal kidney cortex tissue, a primary normal kidney cell line, and a plasma sample are shown. Proteins were separated using a pH 3.0–10.0 nonlinear immobilized pH gradient in the first dimension and 10% SDS-PAGE in the second dimension. Gels are shown acid to basic from left to right and molecular weight markers are indicated.

Performing protein separation on the basis of two independent protein characteristics (pI and molecular weight) allows up to 2000 protein species to be resolved in a standard 18-cm gel (Fig. 2). Using broad-range pH gradients (pH 3.0–10.0) in the first dimension, profiling of only the most abundant proteins in a tissue lysate is feasible. Two strategies can be adopted to allow the study of less-abundant proteins: the use of narrow-range (e.g., a single pH unit) immobilized pH gradient strips for isoelectric focusing (so-called zoom gel technology) (5–8); and sample prefractionation, such as on the basis of differential extraction, chromatography, or subcellular fractionation (9,10). By adopting these approaches, impressive results can be achieved in terms of proteome coverage. Nonetheless, 2D-PAGE does have its limitations, membrane proteins are underrepresented in 2D gels and proteins at the extremes of both pI and molecular weight are difficult to analyze. Furthermore, although in the pharmaceutical/biotechnology industry 2D-PAGE has been adapted for use on a large scale, in an academic setting the technology is not high throughput and generally requires time-consuming image analysis.



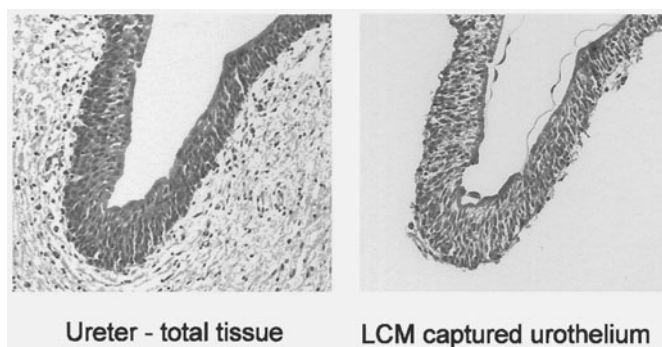


Fig. 3. Laser capture microdissection (LCM) allows selected populations of cells to be collected from stained tissue sections being viewed with an inverted microscope. A cap coated with a thermolabile film is placed in contact with a tissue section. A laser (diameter: 7.5–30  $\mu\text{m}$ ) is used to induce localized melting of the film, causing it to fuse with the underlying tissue. Captured material is then selectively removed when the cap is lifted. Microdissection of urothelium from ureter is shown as an example.

Differential expression profiling aimed at identifying disease markers using a 2D-PAGE-based approach has been applied to the study of many tumor types, including bladder, ovarian, renal, lung, prostate, ovarian, head-and-neck, colorectal, and breast cancers (11–15). An important consideration in such studies is tissue heterogeneity, with relevant cell types often being in a minority or samples containing areas representing different stages in the tumorigenic process. The simplest strategies either have analyzed whole extracts prepared from tissues, such as normal and tumor, with subsequent downstream validation confirming the cellular compartment from which potential biomarkers originate, or have used cell lines as a source of enriched cell populations. Although cell lines are invaluable in many situations, concerns regarding artifacts arising from growth in culture, particularly for established cell lines, have led many researchers to develop alternative strategies to purify cells of interest from tissue samples including antibody-based selections; “nonenzymatic sample preparation” of tumor cell lysates; and laser-assisted microdissection systems, such as laser capture microdissection (LCM) (Fig. 3) (16). A selection of studies illustrating the various approaches is described below.

A study of whole-tissue extracts of renal cell carcinoma and matched normal kidney cortex using 2D-PAGE and silver staining identified 32 proteins that were upregulated in at least four of six samples, including thymidine phosphorylase, annexins I and II, and cofilin (99). It is noteworthy that while thymidine phosphorylase and annexins I and II were expressed by tumor cells, cofilin was found to localize to infiltrating lymphocytes by immunohistochemistry (IHC), illustrating the problems associated with tissue heterogeneity even in samples in which the cell type of interest is relatively prevalent. In addition, this study identified 41 proteins that were downregulated in six of six samples. Thymidine phosphorylase is being studied as a potential vaccine candidate.

A considerable amount of data has been amassed in the systematic analysis of bladder cancer, often using short-term  $^{35}\text{S}$ -metabolic labeling of fresh tissue samples.

By comparing the protein profiles of squamous cell carcinoma and normal urothelium, a number of differentially expressed proteins including several keratins, adipocyte fatty acid-binding protein, galectin 7, stratifin, and psoriasin were identified (17,18). Using antibodies to some of these molecules, serial sections of bladder tissue were labeled to examine the changes in urothelium associated with the development of invasive cancer, a process termed *immunowalking* (18). In addition, analysis of urine samples identified psoriasin as a putative urinary marker for squamous cell carcinoma (19,20). If the false positives seen in women, possibly due to squamous metaplastic conditions of the trigone, can be addressed, psoriasin may be useful in a panel of biomarkers for following patients with squamous cell carcinoma. A similar approach has been adopted for the study of transitional cell carcinoma, with markers selected on the basis of being twofold up- or downregulated in at least 40% of patients with grade 3 T2–T4 tumors compared with normal biopsies (21). IHC of 30 G1 Ta tumors using antibodies to a number of cytokeratins allowed classification of low-grade transitional cell carcinomas into five heterogeneity groups, with a promising preliminary correlation with outcome. This study now awaits long-term follow-up data.

Alaiya et al. (22,23) used a 2D-PAGE approach to compare the protein profiles of benign, borderline, and malignant ovarian tumors with the aim of analyzing the quantitative data using artificial learning strategies to identify a panel of markers for pathologic discrimination of borderline tumors. Purified populations of tumor cells free from contaminating serum and stroma were collected from a series of patient samples and analyzed by 2D-PAGE and silver staining. Spots were selected based on their differential expression among benign, borderline, and malignant samples, and using partial least squares discriminant analysis and hierarchical clustering, promising results were obtained for the identification of borderline tumors. Subdividing the data depending on tumor type (e.g., focusing on serous or mucinous subsets of tumors) may simplify this analysis and improve clustering of tumors with similar clinical outcome.

Since the initial demonstration that 2D-PAGE can be used to analyze LCM-procured samples (24), several cancers have been analyzed using this approach including esophageal (25), prostate (26), and ovarian tumors (27). The issue of sample amount needed obviously limits this strategy; nevertheless, several interesting molecules have been identified. Of particular note was identification of annexin I as a protein lost in the development of esophageal cancer (25). LCM and Western blotting have shown that this loss is an early event in the development of esophageal and prostate cancers (28).

Cell lines are irreplaceable as in vitro model systems for analyzing tumor behavior, such as after drug treatment for studying both the mechanism of drug action and the mechanisms determining relative resistance/sensitivity. As well as growing cells under standard culture conditions, cell lines can be grown as three-dimensional spheroids, which more closely resemble the in vivo tumor environment (29,30). Changes that occurred in MCF-7 cells after treatment with doxorubicin included downregulation of Hsp27 isoforms (31), while daunorubicin treatment of pancreatic carcinoma cells resulted in increased expression of 17 proteins, including the chaperones GRP78, TCP-1, and HSP60; cytokeratins; and Drg1 (32). Thirty-five changes in proteins associated with energy metabolism, cytoskeleton, cell viability, and protein synthesis were discovered after 5'-azacytidine treatment of lymphoma (33), and ionizing radiation upregulated chaperones and downregulated intermediate filament proteins in human prostate epithelial cells (34). Comparison of chemosensitive and resistant melanoma cell lines and chemoresistance or thermoresistance in stomach

cancer cell lines also identified a number of proteins including several chaperones and cytoskeletal proteins (35).

The analyses done to date have generally used whole cell lysates (either from whole tissue or from purified cell populations) and have not exploited zoom gel technology, which is reflected in the nature of the proteins so far identified. The analyses can be explained by the early stage of the field as well as the problems encountered when limited to small amounts of clinical material. However, despite only screening the most accessible fraction of the proteome, interesting findings have been obtained that now await further study. As less-abundant proteins are studied, 2D-PAGE is likely to have a significant impact on the understanding of tumor biology and be applied with increasing success for the identification of tumor markers.

One strategy for studying less-abundant proteins is to focus on particular classes of molecule, such as studying proteins that are subject to a particular posttranslational modification. Phosphorylation is perhaps the most widely studied posttranslational modification in tumor biology. Phosphoamino acid-specific antibodies have become central to the study of global phosphorylation. In a study examining the signal transduction in mouse fibroblasts after stimulation with platelet-derived growth factor (PDGF), 2D-PAGE was used to separate proteins before to immunoblotting for phosphoamino acids (36). Several proteins showing altered phosphorylation were identified. An alternative strategy that has been adopted is immunoprecipitation of phosphoproteins from cell extracts before analysis by 1D- or 2D-PAGE (37–39). Labeling of cells with  $^{32}\text{P}$  is another approach for profiling global phosphorylation. Similar approaches for the analysis of glycosylation using lectins for detection of sugars is an area that will receive increased attention in the near future.

An attractive alternative approach to 2D-PAGE is 2D differential gel electrophoresis, in which two samples to be compared and a reference sample (usually a pool of all the samples in an experiment) are labeled with different fluorescent dyes and then run on a single gel (40). This method has the advantage of simplifying gel analysis, since no gel matching is required and it allows gel-to-gel variation to be dramatically reduced by the use of an internal standard. The use of this technology has been restricted to a small number of studies to date, but promising preliminary results have been obtained, as shown by the analysis of the effects of overexpression of ErbB-2 in a model breast cancer cell line (41) and profiling of LCM-procured esophageal carcinoma cells and normal epithelium (42).

In addition to comparative analyses such as those described above, the application of proteomic technologies also allows approaches to understanding the basis and utility of antibody generation and specificity in cancer patients. Identifying tumor antigens (Ags) that elicit a humoral response, such as differentiation Ag, mutated proteins, and overexpressed proteins, has been the subject of a number of analyses, with SEREX (serologic identification of Ags by recombinant expression cloning) being applied to the study of a number of cancers (43). In the approach, expression libraries are probed with patient sera to identify potential Ags. An alternative strategy combining 2D-PAGE with Western blot analysis using patient sera has been adopted more recently. In this approach, which has been termed SERPA, PROTEOMEX, and SPEAR by various groups, extracts from cell lines or autologous/allogeneic tissue are probed with patient and control sera. Using tissue or cell lines as a source of Ag for screening allows the same repertoire of potential antigens as SEREX to be identified but also allows the study of posttranslational modification-dependent Ag.

In the first study of this kind in the field of cancer biology, a neuroblastoma cell line was probed with sera from 23 neuroblastoma patients, 12 patients with other solid tumors, and 13 controls to look for IgG and IgM reactivity (44). A protein of 50 kDa was identified in 11 of 23 patients with neuroblastomas and 1 patient with a neuroectodermal tumor. This protein was identified as beta-tubulin isoforms I and III. Although reactive sera detected these tubulin isoforms in autologous and allogeneic tumor tissue, they did not react with normal brain tissue.

In a similar study, a lung adenocarcinoma cell line was probed with sera from 54 patients with lung cancer, 60 other patients with cancer, and 61 noncancer control subjects, including 10 with chronic lung disease (45). Sera from 60% of the patients with lung adenocarcinoma and 33% of the patients with squamous cell lung carcinoma were found to react with glycosylated annexin I and/or II. These patients also had higher serum interleukin-6 (IL-6) levels, which may reflect immune system activation and could be responsible for upregulation of annexin I and II. Indeed, a higher membrane-bound fraction of annexins was found compared with patients without reactivity.

This approach has now been extended to a number of other tumors and several further Ags have been identified, including PGP 9.5 in lung cancer (46), RS/DJ-1 in breast cancer (47), and a several Ags in hepatocellular (48) and renal cell carcinoma (49,50). Serum reactivity to these Ags was found in a smaller fraction of patients than for the examples described above, which may reflect tumor heterogeneity, but this does not necessarily undermine their potential role in tumor vaccines.

## Alternative Analytical Techniques

2D-PAGE remains unsurpassed as a high-resolution separation tool for resolving thousands of diverse protein species. With improvements in sample fractionation and solubilization protocols as well as advances in immobilized pH gradient technology, profiling of traditionally inaccessible classes of protein is becoming more straightforward (51–53). However, other complementary technologies have been developed to provide high-throughput alternatives for protein expression profiling, including alternative separation strategies combined with mass spectrometry and arrays.

### **Surface-Enhanced Laser-Desorption Ionization Protein Chip Technology**

Surface-enhanced laser-desorption ionization (SELDI) is an alternative protein-profiling strategy that combines sample prefractionation with mass spectrometry (54). Proprietary protein chip technology allows the affinity-based selection of proteins from a sample of interest using standard chromatographic separation moieties, such as anion and cation exchange, reverse phase, normal phase, and immobilized metal affinity capture. A spectrum (i.e., a profile of intensity vs mass/charge [ $m/z$ ]) of the ionizable protein(s) bound is then generated by time-of-flight mass spectrometry (Fig. 4). SELDI is a highly sensitive technique that is ideal for the study of low-molecular-weight protein species (<20 kDa) that tend to be excluded from 2D-PAGE analysis. Furthermore, the data obtained in a SELDI experiment are well suited to bioinformatic analysis with the aim of identifying discriminatory protein patterns or signatures with clinical utility. Such profiles have the potential to be used directly in the screening and diagnosis of cancer, as well as in classifying different prognostic groups.

SELDI has been used as a profiling tool in the analysis of a variety of tumor tissues, with LCM being frequently used to generate enriched samples for analysis. These

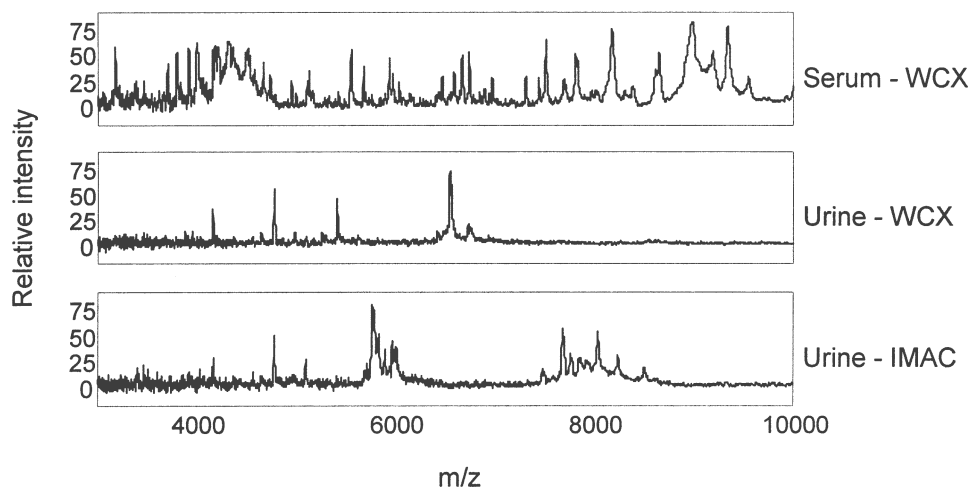


Fig. 4. SELDI profiling of biologic fluids. Representative spectra of serum analyzed using a weak cation-exchange protein (WCX) chip and urine analyzed using a WCX protein chip and an immobilized metal affinity capture protein chip (IMAC) are shown.

studies have indicated the potential of using SELDI to generate tissue- and tumor-specific spectral profiles, with preliminary comparative analyses giving promising results (55–60). However, the studies that have shown the real potential of SELDI have been in the profiling of biologic fluids, including urine (61), serum (62–66), nipple aspirates (67,68), and pancreatic juice (69), using downstream analysis to generate tumor-specific patterns.

In an exploratory study looking for a diagnostic tool for bladder transitional cell carcinoma, urine samples from 30 patients with transitional cell carcinoma, 30 patients with other urogenital diseases, and 34 healthy control subjects were analyzed using a strong anion-exchange protein chip (61). The presence/absence of five potential biomarkers and seven protein clusters was found to show statistically significant differences between urine from patients with transitional cell carcinoma and control urines, with four biomarkers and six clusters remaining significant when the control group of patients with other urogenital diseases was taken into account. With the exception of one protein cluster at 79.5–82 kDa, all the discriminatory peaks were associated with transitional cell carcinoma. Combinations of these individual features were compared to develop a biomarker panel, with the most promising, using biomarkers at 3.3/3.4 kDa and 9.5 kDa and the 85- to 92-kDa protein cluster, achieving good overall sensitivity (87%). Moreover, this biomarker panel gave a sensitivity of 78% for low-grade tumors ( $n = 9$ ), significantly better than results obtained with urine cytology. Although this study is preliminary and gave poor specificity (66%), it clearly highlights the potential of SELDI as a profiling tool and has been followed by a number of similar studies, many incorporating computer-based pattern analysis to maximize the information extractable from the data.

Petricoin et al. (62) analyzed the serum of 50 patients with ovarian cancer and 50 control subjects (including women with benign ovarian cysts) from a high-risk group using samples from the National Ovarian Cancer Early Detection Program (62). Pro-

teins under 20 kDa were profiled using a hydrophobic H4 protein chip. Genetic algorithms and cluster analysis were used to develop an optimum discriminatory pattern that was able to distinguish neoplastic and nonneoplastic samples. Using a blind test set, 50 of 50 patients were correctly assigned to the cancer group including 18 with stage I disease, and 63 of 66 control subjects were recognized as being in the noncancer group, including 17 with benign gynecologic disease and nongynecologic inflammatory disorders. Other similar studies using a range of pattern recognition strategies have been reported, including analyses of serum to identify profiles for detecting breast and prostate cancers (63–66), with one study using a boosting decision tree algorithm achieving sensitivity and specificity of 100%.

It will be interesting to see how many of these reported discriminatory patterns stand up to further testing, with larger blind test sets being analyzed over time and clinically relevant control groups being included to test a range of nonmalignant conditions that are routinely encountered and likely to present with symptoms similar to a particular cancer. More technical points, such as aspects of sample processing and stability, the need to normalize for protein load before analysis, and data manipulation for normalization, quantitation, and automatic peak detection, will need to be addressed to assess the robustness of such approaches in future clinical practice.

Although clinically relevant information can be extracted directly from SELDI spectra using sophisticated pattern-recognition algorithms, ultimately it is a requirement to identify peaks of interest both to understand disease pathogenesis and to enable development of sensitive immunoassays. This has been most frequently achieved using antibodies following predictions based on protein size and knowledge of the biology of the system under study. This is illustrated by identification of the 3.3- to 3.4-kDa biomarker used in discrimination of bladder transition cell carcinomas as defensin (61) and the 16.6-kDa peak identified in comparisons of pancreatic juice from patients with pancreatic ductal adenocarcinoma with patients with other pancreatic disorders as hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I (69). On-chip purification can be done followed by on-chip digestion and peptide mass fingerprinting, and for  $m/z$  species <3000, laser desorption/ionization–quadrupole–quadrupole time-of-flight tandem mass spectrometry has been developed to allow collision-induced dissociation and generation of protein sequence data. Use of these approaches has resulted in the identification of a 5.8-kDa peak upregulated in prostate cancer as a fragment of semenogellin (human seminal basic protein) (54). In cases in which this is not possible, larger-scale purification strategies are required. The feasibility of achieving this aim was initially shown by the identification of virulence factors in *Yersinia pestis* (70). The recent identification of major urinary protein from whole lysates of mouse liver in a study on peroxisome proliferation (71) illustrates that this is possible with more complex protein samples. It is likely that many of the publications reporting descriptive comparative analyses of tumor development will now be followed by protein identifications.

### **Other Protein Separation and Mass Spectrometry-Based Approaches**

To overcome some of the limitations of 2D-PAGE, including throughput and proteome coverage (particularly to improve the study of membrane proteins), several groups have preceded mass spectrometry with alternative protein separation strategies such as liquid-based isoelectrofocusing by free-flow electrophoresis followed by 1D SDS-PAGE. In one study, the membrane fraction of a colorectal cell line was separated by nonreducing 1D SDS-PAGE, and gel slices were subjected to tryptic digestion fol-

lowed by reverse-phase high-performance liquid chromatography tandem mass spectrometry, allowing identification of over 284 protein species, including 92 membrane proteins (72). Multidimensional chromatography (strong cation exchange followed by reverse-phase chromatography) has been adopted, as illustrated by analysis of the *Saccharomyces cerevisiae* ribosome complex (73) and an *Escherichia coli* cell lysate (74). This technique has a 10-fmol detection limit, which can challenge the sensitivity of silver staining. Although these mass spectrometry-based techniques can achieve impressive results in terms of defining a proteome, they are at best semiquantitative.

Isotope-coded affinity tag (ICAT) technology uses mass spectrometry to quantify and analyze protein peaks (75). ICAT reagents consist of a biotin moiety for affinity purification and a thiol-specific reactive group separated by a linker that can be in a light (d0) or a heavy (d8) form, depending on the substitution of eight hydrogens with deuterium. Protein extracts to be compared are labeled on cysteine residues using either the light or heavy ICAT reagent, the mixtures are pooled and digested, and then biotinylated peptides are purified by avidin-based affinity chromatography and analyzed by mass spectrometry. Each peptide will be visualized as two peaks separated by 8 Da in the resulting spectrum, with areas under the peaks representing the relative peptide levels in the two samples.

ICAT technology was initially applied to a model yeast system comparing growth on different carbon sources (75). In further studies, microsomes were prepared from the myeloid leukemia cell line grown in the presence or absence of phorbol myristate acetate (PMA), which induces differentiation (76). Peptides were labeled, separated by multidimensional chromatography, and analyzed by tandem mass spectrometry. In this study, 491 proteins, including many membrane proteins, were identified and several significant changes in relative protein levels were discovered. By using an alternative mass spectrometer, quantitation followed by identification of differentially expressed peptides can be achieved, rather than identifying all peptides in the sample. This was illustrated by the analysis of differentially expressed secreted proteins from nontumorigenic and cancerous human epithelial prostate cell lines, with 33 differences being noted in the spectra and eight proteins subsequently identified (77). ICAT technology is likely to become established as a more routine tool for proteome analysis.

An extension of ICAT technology has been described to facilitate the study of phosphorylation by mass spectrometry. Phosphoprotein ICATs (PhIAT) for phosphoserine and phosphothreonine have been developed that incorporate light (d0) or heavy (d4) versions of a biotin-based tag at phosphoserine and phosphothreonine residues (78). Preliminary data using a yeast model system indicate the potential of the approach. Two other groups have chemically modified phosphoamino acids, including phosphotyrosine in peptides and proteins, and these methodologies are also compatible with incorporating ICAT quantitation (79,80).

### **Array-Based Strategies**

The first category of protein arrays, capture or protein-detecting arrays, is a high-throughput alternative for differential expression profiling, designed to match the scale of experiments possible at the mRNA level. Antibodies are the most obvious high-affinity and high-specificity protein ligand for use in such approaches, but arrays can also use peptides, protein or nucleic acid aptamers, and small molecules.

An array containing 368 antibodies to proteins, including intracellular and extracellular matrix proteins, signaling molecules, cell-cycle proteins, growth factors, and

growth factor receptors, was constructed and used to profile samples representing progressive stages of squamous cell carcinoma of the oral cavity (81). Approximately 2500–3500 cells of six cellular compartments (epithelium and stroma from areas of normal, carcinoma in situ, and invasive cancer tissue) were collected by LCM. Protein extracts were biotinylated and used to probe the antibody array with detection using avidin/alkaline phosphatase and chromogenic-substrate development. Samples were analyzed in quadruplicate and 11 differentially expressed proteins were identified, including increased levels of retinoic acid receptor in the stroma surrounding progressing epithelium. These changes were confirmed qualitatively by Western blotting and IHC.

In another study using protein mixtures labeled with spectrally resolvable fluorophores (Cy3 and Cy5), a detailed assessment of competitive probing of antibody arrays was done using 115 commercially available antibody/Ag pairs (82). By comparing different samples to a reference using proteins at known concentrations, the sensitivity, linear dynamic range, and reproducibility of this technology were rigorously tested. This study highlighted the need for antibody validation and careful control of labeling reactions, but the results were promising and a detection limit of 20 pg was achieved for the best antibody-Ag combinations.

Antibody arrays specifically designed for analysis of cytokines using a sandwich enzyme-linked immunosorbent assay (ELISA) type of assay have received the most attention to date. In one study, eight Th1 and Th2 cytokines were analyzed, with the technique achieving comparable or higher sensitivity than standard ELISA assays (83). Huang et al. (84) developed arrays to measure multiple cytokines, chemokines, and growth factors initially using membrane-based arrays and more recently using a cytokine chip. In one study examining the mechanism of growth suppression of a glioblastoma cell line in response to connexin 43 expression, 43 cytokines were analyzed and monocyte chemotactic protein-1 was found to be significantly downregulated. The development, validation, and use of cytokine chips for the simultaneous analysis of multiple cytokines, such as in sera from patients with cancer, are likely to receive considerable attention in the near future.

Although antibody arrays have been developed on a relatively small scale to date, the possibilities are striking. Development of a large repertoire of suitable antibodies and other ligands for construction of much larger arrays is now an essential requirement for the scope of the technology to be fully tested. Antibody arrays have the potential to be used to examine posttranslational modifications. Probing proteins bound to antibody arrays with phosphoamino acid-specific antibodies may be used to examine phosphorylation status of proteins; similarly, lectins can be used to examine protein glycosylation.

In the second category of protein array, peptides or proteins can be displayed and the arrays used for analyzing aspects of protein function. The use of such arrays for screening for protein–protein interactions or for targets for drugs or small molecules has been examined (85,86). In the most comprehensive study to date, proteins encoded by 5800 open reading frames in the yeast *S. cerevisiae* were expressed with an N-terminal GST-his tag, purified and arrayed in an attempt to generate a proteome microarray (87). This array was screened for interactions with proteins and phospholipids. Although such an array is biased against certain classes of protein, such as those with N-terminal signal peptides where the tag will interfere with correct protein trafficking and processing, the possibilities for generating data on protein–protein interactions are enormous. The



ability to array membrane proteins has been demonstrated (88). The potential use of peptide/protein arrays for identifying substrates for protein kinases, either using  $^{32/33}\text{P}$ -labeled adenosine triphosphate in the kinase assay or using detection systems based on the use of antiphosphoamino acid antibodies, has also been shown (85,86,89,90), and arrays have been used to look for cell adhesion (86).

Protein arrays are an obvious tool for screening for antibodies in patient sera as another alternative to SEREX or 2D-PAGE and Western blotting-based approaches. This strategy has not been used to analyze sera from cancer patients to date, but it has been successfully adopted in screens for autoantigens (91,92). One study probed 196 proteins, peptides, and biomolecules arrayed on a glass slide with well-characterized sera from 50 patients with a number of autoimmune diseases, using a fluorescent secondary antibody as a detection system (92). The results correlated precisely with more conventional assays, giving a strong endorsement for the approach. The technology has been applied to allergy and the diagnosis of infectious diseases (93,94). The use of arrays based on expression libraries, such as the UNIClone set from a fetal brain cDNA expression library (95), as tools for detecting cancer-specific antibodies is likely to be a fruitful approach.

Arrays of cellular fractions or LCM-procured samples with antibody-based detection (so-called reverse arrays) (96) are also likely to be used increasingly, giving a high-throughput alternative to Western blotting, similar to the use of tissue arrays (97,98) as a high-throughput alternative to IHC.

## Conclusion

Proteomics offers a powerful approach to the study of cancer biology, however, the technologies remain demanding with critical issues to address in the preparation of samples and the validation of results. Although it is too early for the potential of proteomics to be fully realized, from the promising results that have been obtained by application of more-established technology as well as in the development of high-throughput novel approaches, it is easy to see the possibilities that are now being offered. With complementary techniques for extensive global protein profiling, there is now a real possibility for investigating the pool of potential markers and targets available at the protein level, with the specific study of posttranslational modifications being an area of research that is likely to feature increasingly. Downstream validation studies are now required to follow up the investigations that have been published to fully assess their potential impact, with the real hope that over the next few years there will be the prospect of results translating into clinical use.

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# II

## DISEASED REGULATORY PATHWAYS

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# 9

## Growth Factor Signaling Pathways in Cancer

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**Daniel Kalderon**

### Introduction

Components of growth factor–signaling pathways were among the earliest gene products implicated in cancer induction in animals and then inferred to be instrumental in human cancers (1,2). This discovery led to the general idea that inactivation of tumor suppressor genes (TSGs) or activation of protooncogenes by mutation in cancer cells substituted in some way for the normal environmental cues that regulate cell proliferation and function. This chapter compares the effects of normal environmental stimuli on cells with those of oncogenic mutations. Superficially at least, some of these comparisons provide a compelling explanation for why certain combinations of mutations are likely instrumental in promoting three fundamental behaviors of cancer cells—cell growth, cell division, and cell survival. Scientists only have a partial understanding of the links between specific mutations and cell behaviors, however. For example, it is not generally understood why some mutations are oncogenic only for some cell types, whether requisite or preferred orders of accumulation of specific mutations exist in various forms of cancer, and what are the key target cells that give rise to cancers. To approach these issues, it is essential to consider and study the role of signaling pathways in context, both during normal development and in carcinogenesis.

### Molecular Pathways for Regulating Cell Division, Cell Growth, and Apoptosis

This chapter first reviews what is best understood—the behavior of isolated cells in a controlled, extracellular environment—and then discusses concepts and questions relevant to the development of normal and cancerous cells. The molecular components of a cell cycle have been well described, as have mechanisms for cell-cycle responses to some families of extracellular signals. This information provides a reasonable understanding of how extracellular signals and specific gene mutations stimulate cell cycling. Cell division must be coupled to cell growth (an increase in cell mass) to sustain cell proliferation. Signaling pathways and specific gene mutations that affect cell growth are known, but a clear understanding of mechanisms that couple growth and division or of how signaling pathways achieve the appropriate balance

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between stimulation of cell growth and cell division is still lacking. Mutational stimulation of the core cell-cycle machinery and some of the molecules and pathways that normally activate cell cycling also provoke apoptotic cell death. Some of the signaling pathways and mutations that can counteract those apoptotic signals in normal cells and cancer cells are known. Thus, at least for some cell types in culture, it is possible to describe how a small number of signaling pathways or mutations can promote cell growth, division, and survival.

### **Cell-Cycle Regulation**

In many cell types, decisions about entry into the cell cycle are made at the transition between G1- and S-phase and can be influenced by extracellular signals (3,4). This paradigm has been intensively studied for mammalian fibroblasts and epithelial cells in controlled culture conditions, generating pleasing models that connect several molecules at the heart of the cell-cycle machinery. The central foci are E2F family transcription complexes, cyclin-dependent kinases (CDKs), and the inhibitors of cyclin-dependent kinases (CDKIs) (5–7). E2F proteins and their obligate DP1 protein partners bind to specific DNA sequences and associate in a regulated manner with retinoblastoma (Rb) family proteins to either activate or repress transcription (8,9). CDKs consist of catalytic subunits, which must be activated by binding to regulatory (cyclin) subunits and which can be inhibited either by phosphorylation or by stoichiometric association with inhibitor proteins, CDKIs (10). In mammals, D, E, and A are the principal CDKs, designated according to their regulatory subunits, that are relevant to the G1/S-phase transition. Additional CDKs control passage from G2 into mitosis.

In resting or early G1 cells, both cyclin D-dependent kinases (cyclin D1–3/Cdk4, or Cdk6) and cyclin E-dependent kinases (cyclin E1–2/Cdk2) are largely inactive because of a stoichiometric excess of CDKIs. One family (Kip/Cip) of CDKIs (p27Kip1, p57Kip2, and p21Cip1) binds to the cyclin component of D- and E-type kinases within a kinase complex (11). These CDKIs are potent inhibitors of cyclin E kinases but only inhibit cyclin D kinases at higher concentrations. Indeed, low concentrations of these Kip family CDKIs actually promote assembly and stability of cyclin D kinases (12,13). The other recognized family (INK4) of CDKIs (INK4a–d or p16, p15, p18, and p19, respectively) bind only to Cdk4 and Cdk6 (not Cdk2), preventing association with cyclin D, and thereby selectively inhibiting cyclin D kinases.

Rb family proteins are key targets of CDKs for stimulating the G1/S-phase transition. As a consequence of keeping CDKs inactive in quiescent or early G1 cells, Rb family proteins (Rb, p107, p130) are minimally phosphorylated. In this state, they bind to E2F repressor proteins (E2F4, E2F5), histone deacetylases, and chromatin remodeling complexes to repress transcription of a large number of genes, including some necessary for instigating DNA replication (8). When cells are stimulated to proliferate, cyclin D-, E-, and A-dependent protein kinases are sequentially activated and Rb family proteins become hyperphosphorylated, leading to reduced repression of genes regulated by upstream E2F-binding sites, together with activation of target genes through binding of E2F activators (E2F1–3).

The activities of CDK, CDKI, and E2F complexes are linked so that ordinarily cyclin D-dependent kinase activity responds cumulatively over time to mitogenic stimulation, triggering cyclin E kinase activity at a critical time, followed by positive feedbacks that fully activate cyclin E-dependent kinase and convert complexes at E2F-binding sites fully from repressors to activators (8,9,14).

Cyclin D kinase activity increases through accumulation of larger amounts of each subunit, enhanced assembly of complexes, or reduction of CDKI activities, with two consequences. First, cyclin D kinase phosphorylates Rb proteins leading to derepression of some genes, including cyclin E and Cdk2 (14). Second, Kip family CDKIs bind the increasing number of cyclin D kinase complexes, thereby reducing the availability of Kip to inhibit cyclin E kinases (9). Both of these effects increase cyclin E kinase activity, leading to further phosphorylation of Rb proteins, and to more extensive derepression and activation of E2F target genes, including cyclin A, cyclin E, and Cdk2, completing a positive feedback loop (15,16). Cyclin E kinase also phosphorylates p27, leading to enhanced p27 degradation and increased cyclin E kinase activity (17), further accentuating positive feedback. The net result of these interactions is that cyclin D kinase activity acts as a sensor of mitogenic stimuli over time, building to a threshold, which then rapidly translates to full activation of cyclin E kinase, cyclin A kinase, E2F-responsive genes, and entry into S-phase. Rb family proteins are important targets of cyclin E kinase activation, as described above but less well characterized, additional actions of cyclin E kinase are also essential for transition into S-phase (10).

Many other cell-cycle regulatory mechanisms can affect cell proliferation, such as arrest at the G1/S-phase or G2/M-phase transition in response to activation of DNA damage or mitotic checkpoints. The activation of CDKs requires dephosphorylation by Cdc25 phosphatase, which can be rate limiting, especially at the G2/M-phase transition. Regulation by checkpoint controls and through Cdc25 may be crucial to consider in the context of normal development and cancer development, but are not of primary relevance for the regulation of cycling of most mammalian cells in tissue culture by environmental factors.

### **Regulation of Cell Proliferation by Mitogens**

Many extracellular mitogens have been described for cells in culture. Most prominent among these for fibroblast and epithelial cells are polypeptides such as epidermal growth factors (EGF), fibroblast growth factors (FGF), and platelet-derived growth factors (PDGF) that activate receptor tyrosine kinases (RTKs) (18). Since RTK pathways are a central focus in cancer studies, they are described first, and the relationships among cell division, cell growth, cell survival, and RTK pathways are examined in some detail. How other types of mitogen and contacts with the extracellular matrix (ECM) can augment and access the same downstream pathways is summarized, followed by a presentation of the other major cellular signaling pathways. In most cases, ligands, receptors, signaling molecules, and transcription factors can be categorized into families. For simplicity, a single family member is used unless different family members have disparate activities that are crucial for the point under discussion.

#### **RTK Signaling Pathways**

A large number of growth factors, including PDGF, EGF, FGF, nerve growth factor (NGF), and transforming growth factor- $\alpha$  (TGF)- $\alpha$  family members, signal by inducing dimerization and hence activation of receptors that are protein tyrosine kinases (19). Intermolecular receptor autophosphorylation induced by proximity stimulates enzymatic activity and creates binding sites for signaling molecules that contain Src-Homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (20–22) (Fig. 1). Each SH2 domain recognizes phosphotyrosine itself and a short amino acid sequence C-terminal to the TYR that determines the specificity of SH2–phosphotyrosine bind-

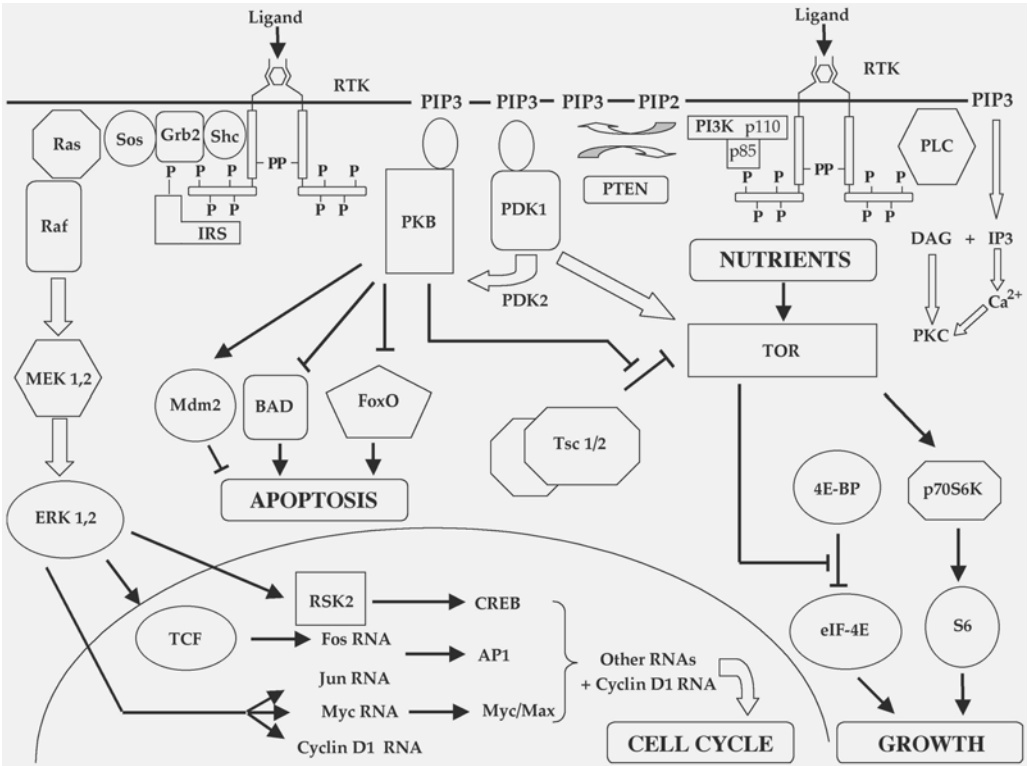


Fig. 1. Major RTK signaling pathways. Ligand binding induces receptor dimerization and autophosphorylation (P), which creates binding sites for several molecules with SH2 or PTB domains. This allows membrane recruitment of Grb2 (directly or through Shc) and Sos, causing activation of Ras, Raf, MEK, and ERKs. Other key recruited molecules are PLC, which becomes phosphorylated and activated, and PI3K, which is brought to its substrates at the membrane. Other recruited molecules, such as IRS, are phosphorylated on tyrosine residues and recruit more molecules with SH2 or PTB domains. PI3K produces PIP<sub>3</sub> at the plasma membrane, leading to membrane localization and activation of PDK1 and PKB. PKB promotes cell survival through at least three targets: Mdm2, BAD, and FoxO family transcription factors. PDK1 and PKB also activate the TOR kinase, which additionally responds to nutrients, and thereby increases translation of mRNAs, especially those with oligopyrimidine tracts or secondary structure in the 5' UTR. ERK activation induces immediate early genes such as fos and myc. Fos and Myc contribute to transcription complexes that, along with the CREB family, induce secondary changes in transcription. A key response for cell-cycle induction is thought to be increased cyclin D1 transcription. Several additional responses to RTK pathway activation are not shown here but are included in Fig. 2. TCF, ternary complex factor.

ing (23). Phosphotyrosine-binding domain recognition of phosphotyrosines depends on the nature of the 3–5 N-terminal amino acid residues (24). In some cases, most notably for insulin-like growth factor (IGF) and FGF receptors (IGFRs and FGFRs, respectively), docking proteins (insulin receptor substrate [IRS] or FGF receptor substrate [FRS] for IGFRs and FGFRs, respectively) bind to the phosphorylated receptor, becoming phosphorylated themselves, providing several additional PTB sites (Fig. 1).

RTKs (plus associated docking proteins) have variable numbers and contexts of TYR residues that are phosphorylated after activation, and can recruit different subsets of SH2/PTB domain molecules. The PDGF receptor (PDGFR) has 12 sites and can bind many SH2 domain-containing proteins including phospholipase C $\gamma$  (PLC $\gamma$ ), cytoplasmic TYR kinases of the Src family, the p85 regulatory subunit of phosphatidylinositol 3' kinase (PI3K), the protein TYR phosphatase SHP-2; the adapter proteins Grb2, Shc, Nck, and Grb7; and the Ras guanosine 5'-triphosphatase (GTPase) activating protein GAP and transcription factors of the STAT family (25,26). In each case, binding to the receptor activates the recruited signaling molecule through an allosteric change, by TYR phosphorylation, by apposition to binding partners or substrates at the membrane, or through a combination of these mechanisms (20). Hence, ligand binding to a single type of RTK can activate many types of molecules at the plasma membrane (Fig. 1). This binding could produce a large number of diverse responses. However, many of these receptor-bound signaling molecules feed into common pathways, so that the net outcome of RTK signaling is not completely bewildering. Indeed, the immediate pattern of transcriptional changes initiated by the various branches of RTK signaling are quite similar (26). It is nevertheless crucial to consider both the independent contribution of each RTK pathway and their cumulative diversity because there are many consequences of RTK pathway activation beyond immediate changes in patterns of gene transcription.

#### RAS/ERK PATHWAY

The most prominent RTK-signaling pathway is the Ras-ERK (extracellular signal regulated kinase) pathway (Fig. 1). p21-ras is associated with the plasma membrane as a result of farnesylation and is activated by binding guanosine 5'-triphosphate (GTP), a process catalyzed by GTP/guanosine 5'-diphosphate (GDP) exchange factors, such as Son-of-sevenless (Sos) (27). Sos is brought to its substrate by association with the adapter protein Grb2 that binds activated RTKs such as PDGFR and EGFR (20). The adapter protein Shc also can stimulate this process. Shc can bind receptor phosphotyrosines and can be phosphorylated by RTKs or by associated Src-family TYR kinases. Phosphorylated Shc provides binding sites for the SH2 domain of Grb2 and recruits Sos to the membrane. RTKs for insulin and IGF-1 and IGF-2 do not have activation-dependent binding sites for Grb2 or Shc, but they can bind and phosphorylate IRS-1 and IRS-2, which provide phosphotyrosine-binding sites for several molecules including Grb2 and Shc.

GTP-bound Ras recruits the SER/THR protein kinase Raf to the plasma membrane, where it is phosphorylated and activated (28,29). Although Ras can bind directly to Raf, the activation of Raf involves several additional proteins and is not completely understood (27,30). Raf, in turn, activates another protein kinase, MEK, by phosphorylation. Activated MEK phosphorylates the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 by dual phosphorylation of THR and TYR residues. The Raf-MEK-ERK phosphorylation cascade is one of several MAPK phosphorylation cascades in mammalian cells (31–33). In general, the first two components of these cascades have very restricted substrate specificity, which may be further restrained by association with scaffold proteins (34,35). The terminal MAPK component, on the other hand, is generally considered the final effector of the signaling pathway. ERKs can phosphorylate membrane-associated and cytoplasmic proteins. ERKs translocate to the nucleus and phosphorylate transcription factors of the ternary complex factor (TCF) and Ets families, leading to activation of immediate-early genes including *fos*, *jun*, and *myc*,

which also encode transcription factors (36). Transcriptional changes are important mediators of the mitogenic actions of Ras.

Activation of the *c-fos* promoter depends in part on a binding site recognized by a complex of serum response factor and a TCF (Elk-1, Sap-1, Sap-2) that has an Ets-family DNA-binding domain. Phosphorylation of TCF at multiple sites by ERK stimulates transcriptional activation (36). Mitogen stimulation of the *c-fos* promoter is further enhanced by the action of the transcription factor cyclic adenosine monophosphate (cAMP)-responsive binding protein (CREB) at its cognate site. CREB can be activated by a number of protein kinases, including RSK2, which can itself be activated by ERK (37). Other promoters have not been studied as intensively as *c-fos*. The *c-myc* promoter is important but not well understood. It includes an E2f/Ets site and, accordingly, in some cells is inducible by Raf and depends on the Ras/ERK pathway for stimulation by serum (38,39). In other cells, *c-myc* is not strongly induced by Raf but instead is dependent on Src-family kinases and Rho GTPases for induction (40–42).

c-Fos associates with c-Jun to form the transcription factor, activator protein-1 (AP-1) (43). c-Myc associates with Max protein to form a transcriptional activator that binds to “E-box” sites (39,42). Hence, activation of “immediate early” genes by the Ras/ERK pathway is generally mediated by binding sites for Ets and CREB family proteins, whereas delayed immediate-early genes (which respond later and require new protein synthesis for induction) might additionally respond to Myc-Max heterodimers and transcription factors of the AP-1 family through E-boxes and AP1 sites, respectively. It is often difficult to trace the transcriptional effects of Ras/ERK stimulation because many immediate-early genes beyond *fos*, *jun*, and *myc* are induced and because the activity of transcription complexes such as Myc-Max and AP-1 depend very much on the availability of heterodimer binding partners and on the degree of phosphorylation of some of these proteins. For example, the Jun family includes several members, some of which are activated through phosphorylation by another MAPK, Jun N-terminal kinase (JNK), that responds primarily to stress stimuli, but that is also activated in response to several mitogens (43).

The most widely cited target of RTK signaling relevant to cell cycling is the induction of cyclin D1 (9,10). Activation of RTKs, Ras, Raf, or ERKs can activate *cyclin D1* transcription, such that mRNA concentrations increase up to 20-fold, generally peaking about 6 hours after mitogen stimulation (5,44). Furthermore, Ras inhibition prevents increased *cyclin D1* transcription in response to mitogen and inhibits progression to S-phase in an Rb-dependent manner (45). Some evidence exists for direct induction of *cyclin D1* by the ERK pathway through Ets proteins (46). Relevant binding sites in the *cyclin D1* promoter for AP-1 family proteins, and for Myc and CREB family proteins, provide indirect links from the Ras/ERK pathway, which are susceptible to the influence of other pathways (43,46–49). The transcription factors AP-1 and c-Myc appear to be important mitogenic effectors of TYR kinase pathways in some cases since antibodies (Abs) to Fos and Myc can inhibit cell cycling (43). Conversely, overexpression or activated forms of Jun, Fos, and Myc can lead to growth factor-independent cycling.

Despite the discussed evidence for participation of Ets, AP-1, and Myc in some manner, the connection between Ras/ERK and cyclin D1 transcription has been difficult to define precisely and appears not to be a straightforward robust connection. Thus, *cyclin D1* induction generally requires sustained Ras/ERK signaling over several hours; it also requires concerted input from the ECM through integrins and the focal adhesion

kinase (FAK) in anchorage-dependent cells (50,51). Furthermore, other branches of RTK signaling, involving PI3 kinase and Ral-GDS, acting through nuclear factor- $\kappa$ B (NF- $\kappa$ B), can also induce *cyclin D1* mRNA independent of ERK activation (52,53).

#### PI3K PATHWAY

A second major RTK-signaling pathway is that initiated by activation of PI3K (Fig. 1). Recruitment of the p110 catalytic subunit through receptor association of the p85 regulatory subunit stimulates activity, perhaps largely by plasma membrane apposition, close to a source of phospholipid substrates (54). PI3K phosphorylates the 3' position of the inositol residue in phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 3,4 diphosphate (PtdIns[3,4]P<sub>2</sub>; PIP<sub>2</sub>).

The phosphatidylinositol 3,4,5 triphosphate (PtdIns [3,4,5]P<sub>3</sub>; PIP<sub>3</sub>) product derived from PIP<sub>2</sub> phosphorylation is especially important. It stimulates the activation of a number of SER/THR protein kinases including protein kinase B (PKB) and p70 ribosomal protein S6 kinase (p70S6k) (55–57). Activation of these kinases requires a specific phosphorylation within the kinase domain (S308 for PKB) and a second SER or THR phosphorylation C-terminal to the kinase domain (S473 for PKB). In each case, the kinase domain phosphorylation requires a priming step to make the substrate accessible to the protein kinase PDK1 (58). For PKB, the requisite conformational change requires binding of PIP<sub>3</sub> to the pleckstrin homology (PH) domain of PKB and is likely preceded by phosphorylation of S473 (59). S473 phosphorylation is triggered by a PIP<sub>3</sub>-dependent kinase activity, termed PDK2. PDK2 has not been identified molecularly. For p70S6k, conformational priming requires phosphorylation of a FRAP/mTOR site and dissociation from a Tsc1/Tsc2 inhibitory complex.

PDK1 can bind and phosphorylate the kinase domain of all PKC isozymes tested. This binding suffices to activate atypical PKC, but an additional event, such as diacylglycerol (DAG) binding to conventional PKC, is required for activation of other PKC isozymes (57,60). Unlike PKB and p70S6K, activation of PKC can proceed by means that are entirely independent of PDK1. PDK1 itself has a PH domain that is required for PIP<sub>3</sub> to stimulate the phosphorylation of PKC and PKB by PDK1 in vitro. PH domain binding to PIP<sub>3</sub> probably stimulates PDK1 actions by ensuring membrane localization. Hence, generation of PIP<sub>3</sub> by PI3K catalysis contributes through PDK1, PKB conformational changes, and membrane association to the activation of at least three SER/THR kinases, PKB, p70S6K, and some PKC isozymes.

PI3K can also lead to activation of small GTPases of the Rac and Rho families, perhaps by altering the activity of PH domain-containing GTP/GDP exchange factors, such as Vav and Sos (61–64). Rac, Rho, and Cdc42, like Ras, are membrane associated and are activated by catalyzing GTP loading and inactivated by catalyzing GTP hydrolysis. The downstream effectors of the different Ras superfamily members are quite varied. However, two major actions of Rac, Rho, and Cdc42 proteins are regulation of the actin cytoskeleton and transcription through the JNK MAPK cascade (65). Furthermore, PI3K-activated Rac1 and Cdc42 can bind to p70S6K and contribute to its activation, perhaps by targeting to a lipid environment where PDK1 can act (66). Rho family GTPases can substantially modify the temporal pattern of cyclin D1 induction, which, as described above, is thought to be a major outcome of Ras/ERK signaling relevant to the cell cycle (67).

Two of the processes strongly influenced by PI3K activation are apoptosis and cell growth, the latter by way of influencing protein translation (Fig. 1).

Several influences of PI3K activation on translation have been described (68). The most prominent effects center on the p70 S6 protein kinase, the protein kinase TOR, and a family of proteins known as 4E-BP that can bind to the translation initiation factor, eIF-4E. Phosphorylation of ribosomal protein S6 by p70S6K leads to a modest, immediate, and general increase in protein translation but a very large stimulation for a subset of mRNAs that include a polypyrimidine stretch in their 5' untranslated region (UTR) (68,69). Among such mRNAs are those encoding ribosomal proteins and other components of the protein translational machinery, so that the cell's translational capacity subsequently increases significantly.

The binding of eIF-4E to eIF-4G is critical for loading capped mRNAs onto ribosomes (70). eIF-4E binds to the 5' terminal cap structure of mRNAs and to eIF-4G. The scaffolding protein eIF-4G, in turn, binds to the 40S ribosome and to eIF-4A, which collaborates with eIF-4B to unwind secondary RNA structure. 4E-BP competes with eIF-4G for binding to eIF4E, thereby inhibiting translation initiation, but is inactivated by TOR-dependent phosphorylation (71). Phosphorylation of 4E-BP stimulates general translation about twofold but can stimulate translation of specific mRNAs, such as *c-myc*, that contain highly structured 5'UTRs, >20-fold (72). The PI3K pathway appears to regulate TOR kinase activity through the TSC products tuberous sclerosis complex 1 and 2 (TSC1/2). TSC1/2 inhibits TOR activity, perhaps by direct binding, but inhibition is relieved when TSC2 is phosphorylated by PKB (73). The activity of TOR is also regulated by nutrient availability, although the mechanism for this is not clear (68,74). Nevertheless, the consequence of dual regulation of TOR activity is that both PI3K activity and sufficient nutrient supply must be in place for phosphorylation of p70S6K and 4E-BP, leading to an enhanced translational capacity that underlies cell growth.

Most PI3K pathway effects on apoptosis are mediated by PKB (57,59) (Fig. 1). One crucial target of PKB appears to be the Bcl-2 family protein BAD. Bcl-2 and Bcl-XL are survival proteins that promote continued function of mitochondria, preventing cytochrome-*c* release and the formation of caspase 9/ apoptosis protease activation factor-1/ dATP complexes that otherwise could lead to a cascade of caspase proteolysis that effects apoptosis (75). BAD is one member of the Bcl-2-related subfamily that can inactivate the Bcl-2 survival proteins by direct binding. PKB phosphorylation of BAD promotes interaction with a 14-3-3 protein and prevents BAD from binding Bcl-2 or Bcl-XL (76). Thus, BAD-induced cell death cannot be prevented by PKB in the absence of its target phosphorylation site.

A second major target of PKB is the FoxO Forkhead family (AFX, FKHR, and FKHR-L1) of transcription factors (75,77,78). These transcription factors can activate several proapoptotic targets, including Bim, a proapoptotic Bcl-2 family member, and Fas ligand, which can trigger receptor-mediated apoptosis (75). Phosphorylation of FoxO proteins leads, *inter alia*, to association with 14-3-3 proteins and exclusion from the nucleus, thereby blocking transcriptional activity. A third PKB target relevant to apoptosis is Mdm2. PKB phosphorylation of Mdm2 favors nuclear localization of Mdm2 and increased inhibition of p53, which, if active, is capable of inducing several proapoptotic target genes (79,80).

#### PLC PATHWAY

PLC- $\gamma$  bound to an activated RTK can be phosphorylated on TYR and thereby activated to catalyze cleavage of phospholipids into DAG and inositol triphosphate (IP3) (20). Binding of IP3 to specific receptors on internal membranes leads to  $\text{Ca}^{2+}$  release

from intracellular pools, leading to activation of  $\text{Ca}^{2+}$ -dependent protein kinases. The combination of DAG and  $\text{Ca}^{2+}$  activates conventional PKC isozymes. PKC has many targets including transcription factors responsive to mitogens, but it also can enhance activation of the Ras/ERK pathway (81).

#### INTERACTIONS AMONG RTK PATHWAYS

The bare-bones description of individual RTK-initiated pathways gives an impression of independent pathways and independent molecular outcomes. In fact, a large number of potential interactions occur between pathways and several effectors are shared between pathways (Fig. 2). The most important connection is the ability of Ras to bind and activate PI3K (82–85). Even in light of PI3K structural studies, it is not certain to what extent Ras activates PI3K through conformational changes or through recruitment to the membrane (86). Conversely, PI3K has been shown to stimulate Ras activity and may contribute to Raf activation by enhancing phosphorylation by the Rac- or Cdc42-regulated kinase, Pak3 (87,88). The coupling between Ras and PI3K can produce some synergistic activation, but in most physiologic settings it probably does not lead to strong activation of one pathway by the other in the absence of normal cues for induction of both pathways. In other words, it does not make the separate activation of each pathway by RTK redundant. By contrast, mutationally activated Ras does strongly activate PI3K when overexpressed as a transfected transgene (63,89). To what extent this occurs in response to mutation of an endogenous *ras* gene is not clear.

#### EACH RTK PATHWAY HAS MOLECULAR CONNECTIONS TO MULTIPLE CELL BEHAVIORS

Transcriptional induction of *cyclin D1* is a key outcome of Ras/ERK signaling, whereas protein translation (affecting cell growth) and cell survival are emphasized as key outcomes of PI3K signaling. This oversimplified view is intended only as an introduction. Ras/ERK can induce transcription of the CKIs p16 and p21 (14,89), potentially countering its positive effects on the cell cycle through cyclin D (Fig. 2). ERKs can phosphorylate upstream-binding factor to increase RNA polymerase I transcription of ribosomal genes, contributing to increased translational capacity (90). The Ras/ERK pathway influences ribosome function in other ways. Activation of Mnk1-2 (Map kinase signal integrating kinases) by ERK leads to phosphorylation of eIF-4E and increases cap-dependent translation (70,91,92).

Another important connection of the Ras/ERK pathway to both cell cycle and growth control is through post-transcriptional effects on Myc. ERK phosphorylation of Myc at Ser62 significantly stabilizes Myc (93). Myc itself can induce both *cyclin D1* and *cyclin D2* transcription (14), as well as the activating E2Fs (1–3) (14). Myc can induce *cyclin E* transcription and expression of Cul1, a component of the SCF (Skp1, Cdc53, and F-box) ubiquitination complex that directs proteolysis of p27 that has been phosphorylated by cyclin E kinase (94,95). Myc also induces transcription by both RNA polymerase II and RNA polymerase I of several ribosome components and associated assembly and translation factors (92,96). Thus, Ras/ERK signaling can affect the cell cycle in multiple ways and can stimulate an increase in cellular translation capacity that supports cell growth.

Similarly, PI3K activation can have a major influence on the cell cycle (Fig. 2). Among the proteins whose translation is selectively increased by the translational effectors of PI3K activation are Myc and cyclin D1. PI3K also affects the stability of both proteins. In each case, proteolysis is stimulated by glycogen synthase kinase-3 (GSK-3)



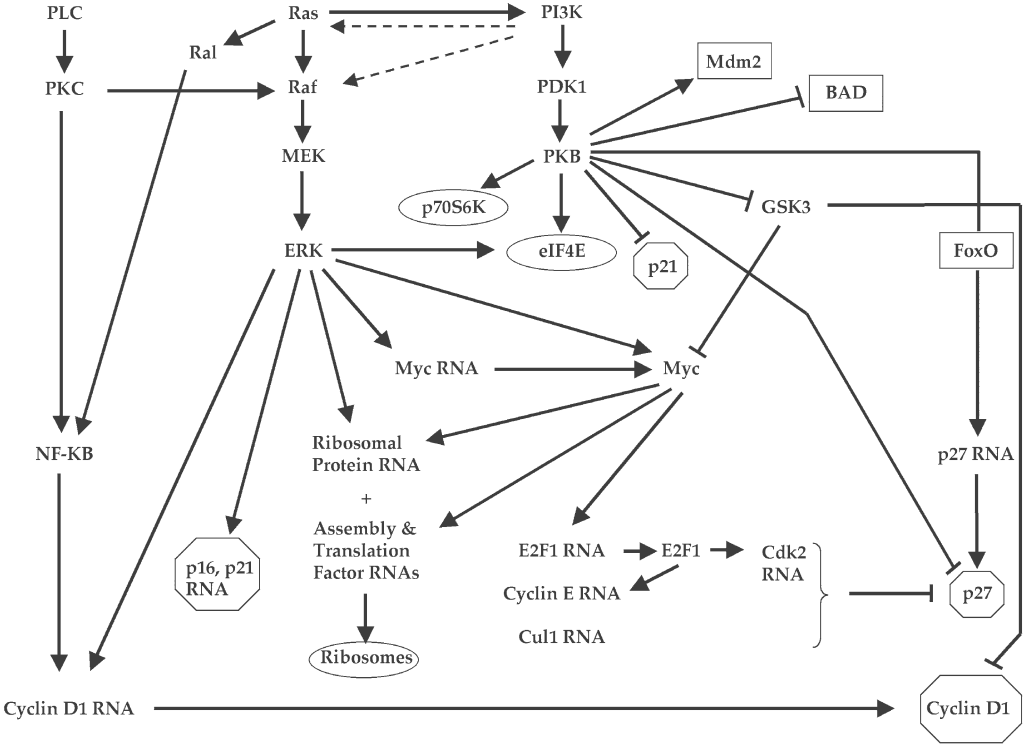


Fig. 2. Connections among branches of RTK pathways. Ras, PI3K, and PLC are activated by RTKs, as shown in Fig. 1. Ras can activate Ral and PI3K in addition to the ERK pathway. Ral activation reinforces induction of cyclin D1 RNA but CDKIs p16 and p21 can also be induced through the ERK pathway. The Ras/ERK pathway also stabilizes Myc (and Fos) protein, accentuating the consequences of inducing the corresponding RNAs. Through both Myc and other transcription factors (including effects on RNA pol I transcription), ERK induces transcription of multiple components of ribosomes and associated assembly and translation factors. ERK also activates eIF-4E through phosphorylation. Hence, the Ras/ERK pathway activates protein translation and growth as well as affects the cell cycle. PI3K acts through PKB to decrease transcription of p27 (through FoxO proteins), and to inhibit the activity of the CDK inhibitors p21 and p27. It stimulates translation of Myc and cyclin D1 through effects on protein translation initiation (as shown in Fig. 1) and stabilizes both Myc and cyclin D1 through inhibition of GSK3 activity. Myc, in turn, promotes transcription of components that will lead to p27 degradation. PI3K can weakly activate the Ras/ERK pathway. Hence, each branch of the RTK pathway affects growth, cell cycle, and cell survival in a variety of ways. Targets key for growth are in ovals, targets key for the cell cycle are in octagons, and those involved in cell survival are in rectangles.

phosphorylation and is inhibited when PKB phosphorylates, thereby inactivating GSK-3 (93,97). PI3K affects the CDKI p27 in several ways. Transcription of p27 is reduced through PKB-mediated inhibition of Forkhead transcription factors (75,78). PKB phosphorylates p27, reducing its nuclear localization and inhibition of cyclin E kinase, while facilitating its proteolysis (98,99). Similarly, PKB phosphorylation of p21 redistributes it to the cytoplasm, reducing its efficacy as a CDKI (100).

The previous description is unlikely to be comprehensive but it is sufficient to make clear that each of the two major branches of RTK signaling can cross-regulate and independently affect cell cycle, cell growth, and cell survival. What are the likely consequences of this arrangement?

First, when RTK pathways stimulate proliferation it is likely that this stimulation is more efficient and effective in many cell types because of the synergism between pathways (such as Ras/PI3K cross-activation, and induction of Myc by both transcription and protein stabilization) and the use of multiple downstream targets (such as increased cyclin D plus reduced p27CDKI to stimulate the cell cycle). Some relevant evidence for this opinion comes from observations of mouse embryo fibroblasts (MEFs). Neutralizing Ab to Ras shows a requirement for Ras to exit G0 and to pass from G1- to S-phase, but the latter requirement is obviated in Rb-mutant MEFs (45,101). Similarly, PI3K was no longer required for passage from G1- to S-phase if Rb was absent, although cells lacking PI3K eventually succumbed to apoptosis (102). Thus, both Ras and PI3K signaling contribute to the G1/S-phase transition through Rb phosphorylation and PI3K contributes to cell survival. Blocking ERK and PI3K activity together blocked proliferation even in Rb null MEFs and was associated with a failure to degrade p27 on serum stimulation (102). This finding suggests that the two pathways have additional collaborative effects on cyclin E kinase activity that are independent of Rb phosphorylation but essential for cell cycling.

Second, although RTK signaling may often produce balanced activation of Ras/ERK and PI3K pathways, there are likely to be several situations (involving activation of certain RTKs), in which a single pathway is primarily activated. In these cases, pathway-specific outcomes will likely be apparent. The predominant effects of Ras/ERK signaling are likely to involve the cell-cycle and cell-fate determination, whereas the major effects of PI3K signaling are likely to be on cell growth and survival.

### *Relationship Between Cell Growth and Cell Cycling*

RTK pathways can stimulate processes that affect cell growth and division, but is it necessary to stimulate both processes independently to stimulate cell proliferation? Normal yeast (*Saccharomyces cerevisiae*) cells achieve a fairly constant size before mitosis over a variety of growth rates. A key mechanism for matching the cell cycle to growth rates appears to be the use of poorly translated mRNA for key cell-cycle components such as the G1 cyclin CLN3 and the Cdk phosphatase CDC25, so that production of adequate amounts of protein to drive the cell cycle requires high translational capacity (ribosomes and accessory factors) in the cell (92,103,104). The coupling between growth and cell division is not so rigid for multicellular eukaryotes (92). Some cells divide without any intervening growth, especially at early stages of embryogenesis, while other cells grow without dividing, as part of their differentiated cell behavior. For cells that do proliferate while maintaining a more or less constant size, we can ask if this is achieved by hardwired molecular connections between growth and the cell cycle or by arranging for independent balanced stimulation of both processes by extracellular signals.

From the limited evidence available, it appears that some growth stimulation is generally required for cell division but that growth stimulation alone cannot drive cell division; conversely, stimulation of the cell cycle seems often to have very little impact on growth (92,105). Thus, some independent stimulation of both cell-cycle and growth pathways will normally be required for proliferation, but the size of proliferating cells may vary depending on the balance of pathways being stimulated. This summary view-

point is illustrated with reference to the developing *Drosophila* wing disc, a growing sheet of epithelial cells. This tissue might, of course, exhibit different regulatory mechanisms from mammalian epithelial cells, but it has been the most thorough testing ground to date for comparing growth and cell-cycle consequences of RTK signaling.

In the *Drosophila* imaginal wing disc, the G1/S-phase transition is limited by cyclin E kinase activity and the G2/M-phase transition by activity of the Cdc25 phosphatase String. Overexpression of cyclin E and String together, or overexpression of E2F, which induces both cyclin E and String transcriptionally, accelerates cell cycles but does not increase overall cell mass; hence, the faster dividing cells are smaller than normal (106). Conversely, overexpression of Rb slows cell cycles and initially does not affect growth, leading to the generation of larger cells (although eventually increase in overall mass is also reduced). This result indicates that passage through cell-cycle transitions does not automatically stimulate cell growth. Overexpression of cyclin D, on the other hand, accelerates cell division and cells retain a normal size and exhibit an unchanged distribution among phases of the cell cycle (107). Thus, cyclin D can affect growth as well as the cell cycle, possibly implicating targets other than Rb as being relevant to growth. If cyclin D affects both cell growth and cell cycle in mammals as well, this would be an important mechanism for ensuring that normal growth factor stimulation affects both cell growth and cell cycling.

What about RTK pathways in the *Drosophila* wing disc? One clear-cut set of results involves the *Drosophila* insulin-receptor pathway, which appears to involve mainly PI3K and downstream effectors without substantial input from the Ras/ERK pathway. Reduced signaling by the *Drosophila* insulin receptor; by the IRS protein, Chico; by PI3 kinase; by PKB; or by S6 kinase produces smaller flies with smaller wings and smaller wing disc epithelial cells (108). In each case (except for S6 kinase), cell division rates are slowed (producing fewer cells), but not as much as growth since cell size decreases. Overexpression of these components or loss of PTEN activity (which normally reverses the phosphorylation of phospholipids by PI3K) produces increases in overall cell mass and cell size. Increased division rates are evident in response to loss of PTEN but not from the other manipulations. One conclusion to be drawn from these results is that the PI3K branch of RTK signaling primarily affects cell growth. Secondly, there appears to be some stimulation of cell cycling, especially from components at the top of the PI3K pathway hierarchy. The cell-cycle effects are associated with shortened G1-phase, implying stimulation of the G1/S-phase transition, consistent with molecular effects on CDKI noted earlier.

The role of the Ras/ERK pathway has not been studied in isolation by manipulating downstream components. Instead, consequences of activated Ras, which also can activate PI3K, have been examined (109,110). Activated Ras increases cell mass accumulation and cell size without accelerating cell division, although G1 is shortened and G2 lengthened. These consequences appear to be attributable largely to increasing amounts of Myc through the Ras/ERK pathway together with activation of PI3K. Increased amounts of Myc alone increase growth without increasing cell division rates (but shortening G1) (111). These results do not discount effects of Ras/ERK and PI3K on the G1/S-phase transition, but they certainly emphasize that both pathways can have a major effect on cell growth. It must be remembered that the effects of signaling pathways and individual components may vary from one cell type to another, from one environment to another, and from one organism to another. The effects of Myc are a case in point. In contrast to the cited *Drosophila* studies, experiments with fibroblasts derived from

Myc-deficient mouse embryos suggest that Myc primarily controls cell cycling and not cellular growth (112).

### *Relationship Between Cell Cycle and Apoptosis*

Apoptosis is an integral part of normal development, whereby excess cells are generated and eliminated if not incorporated into differentiated tissue or the developmental program preceding differentiation. In these circumstances, the cells to be eliminated are often those that fail to make contact with other cells or an ECM presenting cell-survival factors (113,114). This elimination can ensure that different cell types are produced in appropriate proportions. In the immune system more elaborate strategies use apoptosis to eliminate self-reactive cells and to terminate immune responses (115–118). During normal development, cells will often proliferate without any associated apoptosis. By contrast, it is often found that artificial stimuli, such as overexpression of E2F or Myc, which might be expected to induce rapid cell cycling, instead leads to cell death (119). As described below, hardwired connections exist between key stimulators of the cell cycle and apoptosis, but, at the same time, many other factors determine whether the outcome of stimulating these molecules will be proliferation or cell death.

E2F provides a key connection between the cell cycle and apoptosis. Several molecules that are subject to E2F repression or activation are proapoptotic (9,114). One such target is p19ARF (named after the mouse gene; p14 in humans) (120). p19ARF binds to Mdm2, negating the ability of Mdm2 to block p53 activity and induce p53 degradation. p53 induction can activate p21 expression, leading to cell-cycle arrest, or it can promote apoptosis through a variety of other transcriptional targets. Loss of p19ARF (or p53) can steer the consequences of E2F overexpression toward stimulating S-phase in place of cell-cycle arrest and eventual apoptosis, but it does not completely suppress apoptosis induced by E2F or activators of E2F, suggesting that additional mediators of E2F-stimulated apoptosis exist (9,114). Indeed, p73, a relative of p53, is also induced by E2F1 and can promote apoptosis (121). Furthermore, MEFs lacking p73 are more resistant to E2F1-induced apoptosis. APAF-1, a central component of the apoptosome, is also induced by E2F1 and likely to be relevant, as may be several additional targets of E2F (121). Thus, the derepression and activation of E2F-responsive genes that necessarily accompanies entry into S-phase always produces a proapoptotic signal of some magnitude. During normal development, this process is transient and likely to be accompanied by several other molecular changes that include antiapoptotic actions, which prevail. Forced overexpression of E2F does not necessarily have these accompaniments and is sustained.

Of the agents that can indirectly induce E2F activity, Myc is especially proapoptotic, most likely because Myc can directly induce additional proapoptotic targets and because Myc also induces transcription of E2F1, the E2F family member that is the most potent activator of apoptosis (122). Overexpression of Myc can lead to cell-cycle arrest but more often leads to apoptosis. This overexpression can be converted into a proliferative response if accompanied by artificial antiapoptotic signals, such as overexpression of Bcl2, PI3K, or PKB (114,122,123). The outcome of these experiments involving the artificial production of competing pro- and antiapoptotic signals cannot readily be predicted and differs among cell types, presumably because cells are subject to a large number of pro- and antiapoptotic influences that depend on their environment. The overall balance of these inputs appears to be summed by mechanisms involving the Bcl-2 family of proteins and mitochondrial functions to

make a binary decision, much as cell-cycle control arrives at a threshold of stimulating cyclin E kinase activity, beyond which a positive decision becomes irreversible. Cells in some environments may be able to tolerate the proapoptotic consequences of strong Myc or E2F induction, whereas others succumb to apoptosis.

### *Mutational Alteration of RTK Pathways and Their Targets in Cancer*

#### COMMON TYPES OF MUTATION

Regulated presentation of ligands is the key mechanism for restricting the activity of signaling pathways during normal development, so it follows that inappropriate production of ligand could stimulate inappropriate growth, as recognized many years ago in the autocrine growth factor hypothesis (124). During development, the temporally and spatially restricted production of ligands is generally a response to activation of a signaling pathway by another ligand (125). Similarly, the frequent association of growth-factor production with tumors is most often likely to be secondary to internal disruption of a different growth factor–signaling pathway, rather than being caused by mutational alteration of the promoter for the autocrine factor or of specific transcriptional activators or repressors. Inappropriate production of growth factors is common in tumors; it may occasionally contribute to initiation of a tumor (like the *v-sis* gene, encoding a PDGFR ligand, in animal models), but more commonly it will speed further development of cancers (126–130).

Specific mutations affecting receptors can produce ligand-independent dimerization and activation. Such changes include loss of the extracellular region and changes in the transmembrane domain, fusion by genomic translocations of the kinase domain to a protein domain that can dimerize, or even expression of the normal protein at very high levels (20, 131). Inappropriate receptor activation (autonomously or through ligand production) has the virtue of activating all downstream RTK pathways but may not always be effective because of mechanisms of downregulation. Feedback inhibition frequently occurs at multiple sites along a signaling pathway but the receptor is invariably a prime target. For tyrosine kinases, activation frequently leads to receptor internalization and degradation. It can also promote binding of a phosphatase (SHP2) that can dephosphorylate receptor phosphotyrosines, and it can recruit and activate RasGAP, thereby limiting the extent of Ras activation.

The EGFR family (erbB1–4) illustrates some of these principles and includes one member, HER2, that is commonly overexpressed in tumors (132). There are several ligands for the prototypical EGFR, erbB1/EGFR (EGF, TGF- $\alpha$ , amphiregulin, heparin-binding EGF, betacellulin, and epiregulin), as well as for the related receptors erbB3/HER3 and erbB4/HER4 (two families of alternatively spliced neuregulins) but none for erbB2/HER2. ErbB2 can be activated in response to ligands by forming heterodimers with other family members. Indeed, of all receptor isoforms these heterodimers are the most potent activators of downstream pathways and of cell proliferation in tissue culture. Only overexpression of the erbB2 receptor isoform suffices to transform established cell lines. While erbB1 amplification is seen in cancers, there is a particularly strong link between erbB2 overexpression and rapid tumor growth. An activating mutation in the transmembrane region of erbB2 was associated with tumors in rats, and antibodies to erbB2 have been successful in reducing tumor growth in animal studies and clinical trials (125, 132).

erbB2 is particularly potent at stimulating growth for at least two reasons. First, monomeric EGF family ligands have a high-affinity-binding site that dictates speci-

ficity for erbB1 or erbB3/4 and a low-affinity site that binds better to erbB2 than to the other receptors. erbB2 is therefore preferentially incorporated into an activated heterodimer and produces the most stable and persistent signaling complex (133). Second, a homodimeric erbB1 complex with EGF is rapidly internalized and degraded whereas an activated erbB1/erbB2 complex is less rapidly internalized and is recycled to the membrane after internalization (134). Thus, erbB2 overexpression enhances the response of other EGFR family members to normal low amounts of ligand, in part because it is relatively insensitive to negative feedback.

Empirically, Ras mutations appear to be the most common, effective method of activating TYR kinase proliferation pathways through mutation. On one hand, this can be attributed to the design of Ras as an ON/OFF switch whereby specific mutations that compromise GTPase activity leave Ras in a permanent ON state that is inert to negative feedback mechanisms, such as RasGAP activity. On the other hand, Ras occupies a focal point, sufficient to activate the ERK pathway and, when activated by mutation, the PI3K pathway.

PTEN mutations, which result in increased amounts of PIP<sub>3</sub> (as for PI3K activation), are found in a variety of tumors and predispose mice to tumorigenesis (135,136). Whether Ras and PTEN mutations are exclusive or are frequently found in the same tumors has not been reported.

Individual activation of the Ras/ERK pathway downstream of Ras or downstream elements of the PI3K pathway can contribute to oncogenesis, as shown by the transforming retroviruses harboring activated *raf* and *PKB (Akt)* genes, but such mutations are found only relatively infrequently in human tumors. Colorectal cancers and melanomas may present marked exceptions to this generalization. Activating mutations in the B-Raf gene were found in a substantial proportion (almost 10%) of colorectal tumors, especially in those in which mismatch repair was compromised (137–139). In these tumors, mutations in B-Raf were never accompanied by mutations in Ras (present in >50% of tumors), implying that each alteration plays a similar role, presumably through activation of ERK.

Myc is an important effector of RTK pathways that is frequently found to be overexpressed in tumors through chromosomal translocation; gene amplification; or other, more indirect means (122,140–142). Collaboration between activated Ras and overexpressed Myc is seen in cell culture and animal models of transformation and tumor formation (143–146). From what is known about the molecular targets of each, this synergism might reflect a number of interactions; first, the ability of Ras to stabilize Myc protein (93); second, the convergent action of Myc with Ras/ERK and PI3K pathways on cell growth and cell cycle; and third, the antiapoptotic effects of the PI3K pathway countering the proapoptotic actions of Myc (14).

#### CONSEQUENCES OF RTK PATHWAY MUTATIONS

Since oncogenic alteration of Ras-, PI3K-, and Myc-signaling pathways can influence cells in many ways, it is not obvious which particular consequence or consequences are instrumental in the development of cancer. A better perspective on this issue can be gained if we also examine other mutations commonly found in cancer that affect the targets of growth-factor pathways, namely the machinery of the cell cycle and apoptosis.

Mutations that inactivate Rb or p16INK4a, or that lead to overexpression of cyclin D1 or Cdk4, are collectively so frequent in human cancers that it has been suggested that

disruption of normal G1/S-phase regulation is essential to cancer development (9,120). Furthermore, because cancers generally contain only one of these types of aberration (there are exceptions (147)), it is sometimes argued that each such mutation suffices to disrupt G1/S-phase regulation (9). If these suggestions are taken at face value we would need to conclude that Ras, Myc, and PI3K activation do not have a key input into the G1/S-phase transition in cancer cells despite the molecular evidence that they can regulate CDK and CDKI activities in multiple ways. Additionally, the idea that cyclin D1 can be a crucial target of RTK pathways is clearly illustrated in some mouse models. Mice overexpressing Her2 or activated Ras in the mammary epithelium develop breast cancers only if the mice contain a functional cyclin D1 gene (148). Furthermore, the incidence of such cancers is increased in p27Kip heterozygotes (and reduced in p27 nulls, presumably because p27 was required to assemble CDK complexes) (13). Given these contradictory views concerning the connection of RTK pathways and the cell cycle, it is obviously worth considering more carefully exactly what impact common RTK pathway mutations and cell-cycle mutations have on the cell cycle.

Rb, p16, cyclin D, and Cdk4 mutations do not abrogate all control of the cell cycle. p107 and p130, like Rb, can inhibit the cell cycle when overexpressed, and Rb/p107/p130 triple mutant MEFs show much more pronounced acceleration of G1, smaller cell size, and resistance to contact inhibition, serum deprivation, or CDKI expression than do Rb mutant MEFs (9,149). Signaling pathways and internal signals such as DNA damage can regulate the G1/S-phase transition through p107 and p130 in the absence of Rb. The normal patterns of expression of Rb family members no doubt influence which cell types are most compromised for G1/S-phase control by Rb mutations, and therefore which specific cancers are induced by loss of Rb in humans and mice (9,121,150).

Overexpression of cyclin D1 or Cdk4 would affect all Rb family members and could stoichiometrically oppose the influence of all types of CDKI. Amounts of CDK and CDK activity would still be affected by signaling pathways that affect protein translation, complex assembly, and protein stability. Any input that induces CDKI could be of large enough magnitude to delay or prevent the G1/S-phase transition despite high cyclin D1 or CDK4 expression. In other words, there are ways of regulating the G1/S transition even if cyclin D1 or CDK4 are overexpressed.

Loss of p16INK4a would upset the balance between CDKs and their inhibitors in many cell types, but it would be expected to have a major impact only in situations in which high p16 concentrations normally limit cell cycling since other influences, such as regulation of Kip CDKI, can act independently of p16. The importance of p16 inactivation to tumor formation was initially overemphasized because most tumors found to have p16 mutations had also lost activity of p19ARF (which is derived from an alternative reading frame in the same gene) (120). Mice lacking only p16INK4a, as opposed to both p16 and p19, or even p19 alone, have much reduced tumor susceptibility, restricted to a few tissues. INK4c mice are mildly tumor prone with a different tissue specificity (120). Overall, then, it is likely that Rb pathway mutations reduce but do not eliminate the G1/S-phase barrier to cell proliferation, and they probably do so effectively only in tissues in which the gene in question (Rb, cyclin, kinase, CKI) has a high regulatory burden. There is room for RTK pathway mutations to diminish the G1/S barrier further.

If RTK pathway mutations can activate the G1/S-phase transition, why doesn't this suffice? Why is there an apparent need also to dismantle the Rb pathway by mutation? One possible answer is that Ras can induce p16 and p21 inhibitors as well as activate cyclin D1. Hence, removing or reducing Rb function or its regulators, such as p16,

would allow Ras to have a net positive effect on promoting the G1/S-phase transition (89). Thus, Ras and Rb pathways would have complementary, synergistic effects on the cell cycle. Further collaborations with Rb pathway mutations can be suggested. Myc, Ras, and PI3K pathways substantially stimulate cell growth, an essential accompaniment to cell cycling to achieve sustained cell proliferation, whereas both Ras and PI3K mutations can counter the proapoptotic signals that accompany mutational activation of the G1/S-phase transition. The latter antiapoptotic input is not necessarily sufficient because it is well documented that most tumors include disruptions to the p53 pathway (affecting p53 directly, Mdm2, or p19Arf) or to other regulators of apoptosis such as Apaf1 or Bcl-2 family members (9,138,140).

### *Other Pathways That Activate or Augment RTK Pathways*

Activation of RTKs accomplishes many goals involving the regulation of cell fate, cell proliferation, and survival during development. This presumably is why RTK pathway components are present in most cell types and can be altered by mutation to promote cancer development. RTK pathways can be activated or reinforced by a variety of signals that do not directly stimulate an RTK. It is important to be aware of these pathways because they may present additional targets for mutation in cancer and because they can affect the response of cells to other mutations during cancer development.

### GROWTH FACTORS THAT BIND TO G-PROTEIN-COUPLED RECEPTORS

A number of ligands that bind to G-protein-coupled receptors (GPCRs), including lysophosphatidic acid (LPA), neuropeptides (bombesin, bradykinin, endothelin, vasopressin), and other peptides (cholecystokinin, thrombin, gastrin), are primarily known for other functions but can also cause cell proliferation (151,152). Activation of GPCRs activates one or more trimeric G-proteins by charging the G $\alpha$  subunit with GTP and liberating the G $\beta\gamma$  subunits (153). Some mitogenic ligands (e.g., bombesin) activate Gq family proteins, leading to PLC $\beta$  activation and subsequent activation of a MAPK cascade, most likely through PKC phosphorylation of Raf (81,154,155). Ligands (such as LPA or thrombin) that couple to Gi-linked receptors can activate ERK through Ras activation and involve intermediate TYR phosphorylation steps. One possible scenario is that activated G $\beta\gamma$  recruits PI3K $\gamma$  (an isoform that does not associate with p85) to the membrane, resulting in PI3K activation (156). In some manner, PI3K $\gamma$  activity stimulates the activity of an Src family tyrosine kinase, perhaps through direct binding of an Src SH2 domain to PIP<sub>3</sub>, leading to the phosphorylation of RTK (such as EGFR) and Shc that act as adaptor proteins to recruit Grb2 and Sos to the membrane and hence activate Ras (157–159). Ligands that couple to Gs proteins stimulate production of cAMP and activation of PKA. PKA can activate or reduce Ras/ERK activity depending on cell type and available substrates (160). PKA can inhibit activation of Raf-1 by Ras, apparently by a variety of mechanisms, including the intermediate activation of Src and Rap1. Src and Rap1 have been implicated in the mechanisms by which PKA facilitates activation of ERK through activation of B-Raf. Thus, the net effect of PKA on ERK activity depends largely on the relative proportions of Raf-1 and B-Raf.

### EXTRACELLULAR MATRIX LIGANDS

Growth and survival of normal cells in tissue culture is generally dependent on adhesion to a surface that accumulates, or is precoated by, an ECM that includes proteins such as fibronectin, laminin, and collagen (161–163). Many other cell behaviors,



including cell migration or terminal differentiation, frequently depend on interactions with ECM. These diverse contributions of ECM are reminiscent of the actions of growth factors. Indeed, ECM can affect growth factor signaling pathways in several ways (164–166), including binding and retention of growth factors, obligatory associations of heparin or heparan sulfate proteoglycans with FGF to stimulate the FGFR efficiently, and direct transduction of signals by receptors for ECM proteins such as integrin.

An important aspect of integrin–ECM interactions is the concentration of these interactions at a limited number of focal adhesion sites (151,167) (Fig. 3). This concentration is dependent on the activity of Rho family GTPases governing the cytoskeleton and on the cell–ECM interactions themselves for tension (168). A variety of proteins become associated with integrins at focal adhesions, including the TYR kinase, FAK, Src, and the adaptor proteins p130CAS and paxillin. The assembly of this complex is initiated by FAK autophosphorylation, probably due to clustering of integrins in response to binding ligands such as fibronectin or laminin (169). This assembly stimulates further TYR phosphorylations and recruitment of SH2 domain proteins, including Nck, Shc, and Grb2/Sos, leading to Ras activation. Paxillin (indirectly) and Nck can bind to PAK kinases, and interaction with ECM stimulates membrane association of Rac and other small GTPases that can activate PAK. PAK can activate the ERK pathway through phosphorylation of Raf or MEK (170).

A subset of integrins can stimulate Ras through another pathway that is independent of FAK (171,172) (Fig. 3). These integrins are linked to the Src family TYR kinase Fyn by the transmembrane protein, caveolin (173). Integrin ligation stimulates Fyn activity and leads to phosphorylation of Fyn-associated Shc and recruitment of Grb2-Sos and Ras activation. Simultaneous activation of FAK and Shc pathways can also activate Rac, leading *inter alia* to induction of *cyclin D* transcription (174).

Clearly, ECM interactions contribute, largely through Ras/ERK pathway activation, to maintaining cells in an appropriate proliferating or differentiated state and therefore present a key environmental input. Complete loss of ECM contact generally leads to apoptosis, whereas migration of cells to a different ECM environment may affect decisions to proliferate or differentiate.

#### CYTOKINE AND ANTIGEN RECEPTOR PATHWAYS

The development and physiology of blood cells involve a number of signals and receptors that are either unique or are less widely used by other cell types (115–118). Nevertheless, these signaling pathways resemble RTK pathways because they involve a network of protein interactions and culminate in the activation of Ras/ERK and PI3K pathways (175–177). Additional prominent outputs involve transcription factors of NF- $\kappa$ B, NFAT, and STAT families, and sophisticated mechanisms regulate cell behavior in the immune system (177,178). Careful analysis of these signaling mechanisms should reveal a great deal about how interactions among different signaling pathways dictate cell behavior but requires knowledge and explanation of immune system function that is beyond the scope of this chapter. The following discussion is therefore limited to describing the basic hardware of these specialized signaling pathways and some insights into their relationships to cancer.

Hematopoietic growth factors, generally termed *cytokines*, contribute to lineage, differentiation, and proliferation decisions during the maturation of specific blood cell types from a pluripotent stem cell. Proliferation and activation of mature peripheral T

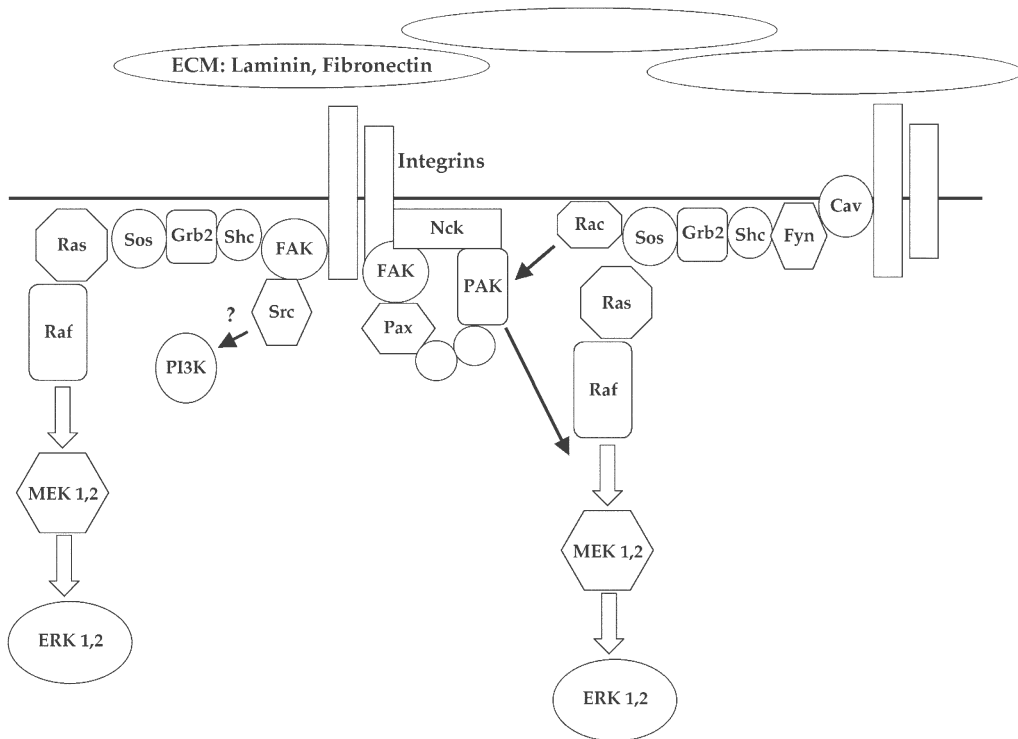


Fig. 3. ECM signaling through integrins. Binding of ligands to integrins can induce assembly of focal adhesion complexes that include the FAK kinase. This can activate the Ras/ERK pathway through recruitment of Shc, Grb2, and Sos. It also can recruit and activate PAKs, which can act on Raf or MEK to augment Ras/ERK pathway activation. PI3K activation through Src is a more speculative consequence of unknown mechanism. It may have the consequence of facilitating Rac activation by Sos. Some integrins can also associate with Caveolin (Cav). This can also activate the Ras/ERK pathway through Fyn tyrosine kinase, Shc, Grb2, and Sos. Although shown in isolation here, integrin-induced pathways are thought generally to augment RTK pathways.

and B cells in response to immune challenge is triggered by binding to antigen (Ag) receptor complexes but additionally depends on signals elicited by cytokines. The B-cell receptor (BCR) and T-cell receptor (TCR) also have essential roles in the early development of lymphoid cells before exposure to specific Ags.

Some cytokine receptors, such as those for colony-stimulating factor-1 (CSF-1) and SCF, are themselves TYR kinases, but a larger class of cytokine receptors triggers TYR kinase phosphorylation events indirectly. Four main families exist within this latter class of cytokine receptors (EpoR, interleukin-2 [IL-2R], IL-3R, IL-6R) that are activated by hetero- or homodimeric ligands, most likely through dimerization (179,180). In each case, a signaling subunit has a sequence motif, known as box1 and 2 close to the plasma membrane and C-terminal TYR residues that become phosphorylated in response to activation. The TYR kinases responsible for phosphorylation are members of the Janus kinase (JAK) and Src families (177,181). The JAKs bind to the box1, 2 region. As with RTKs, receptor dimerization allows initial transphospho-

rylation between JAKs in a receptor complex. This phosphorylation likely activates the JAKs further and stimulates phosphorylation of TYR residues in the receptor and on substrates that become associated with the receptor. At least one receptor phosphotyrosine forms a binding site for a specific member of the STAT family of proteins, which is subsequently phosphorylated by JAK or, in some cases, Src, on TYR residues. This binding triggers STAT dissociation from the receptor and homo- or heterodimerization through mutual interaction of phosphotyrosine regions and SH2 domains. Only STAT dimers can accumulate in the nucleus, bind DNA, and activate transcription.

Although STATs are important effectors for cytokines, JAK activity also leads to recruitment and activation of other signaling molecules, including Shc, leading to activation of the Ras/ERK pathway through Grb2 and Sos recruitment (177,181). Receptor phosphorylation leads to recruitment and activation of PI3K, either through recruitment of IRS proteins or through Shc, Grb2, and Gab2, with phosphorylated Gab2 or IRS directly recruiting PI3K (176). Since members of the Src TYR kinase family including Lck, Fyn, Lyn, Hck, and Syk associate with cytokine receptors, they also can initiate TYR kinase signaling pathways (182).

Stimulation of Ag receptors of T and B cells leads initially to the sequential activation of two families of TYR kinases (Figs. 4 and 5). The exact events that lead to activation of Src family kinases (Lck and Fyn in T cells; Lyn, Fyn, Blh, and Fgr in B-cells) are not clear but are thought to involve intermolecular autophosphorylation and dephosphorylation of an inhibitory terminal phosphotyrosine by the phosphatase CD45, in each case triggered by receptor clustering (183,184). A characteristic motif (immunoreceptor TYR-based activation motif [ITAM]) is then phosphorylated on both TYRs by the activated Src family kinase to form a binding site for an Syk family kinase (Syk in B-cells; ZAP-70 and Syk in T cells). In B and pre-B cells, the ITAM motifs are present on the Ig $\alpha$  and Ig $\beta$  signal-transducing subunits that associate with Ig heavy and light chain responsible for Ag recognition. In T and pre-T cells, the ITAMs reside on CD3 molecules, which associate with the TCR  $\alpha$ - and  $\beta$ -chains responsible for Ag recognition. This association leads to activation of Syk (by autophosphorylation) and ZAP-70 (involving phosphorylation by an Src family kinase). These kinases can, in turn, activate TYR kinases of the Tec family (Itk and Txk in T cells; Btk in B cells) (185).

In T cells an integral membrane protein, linker for activation of T cells (LATS) is a key substrate for ZAP70 that can provide binding sites for SH2 domains of PLC $\gamma$  (which is then activated by Syk and Tec family phosphorylation), p85 of PI3K, and Grb2 (185) (Fig. 5). The adapter BLNK plays a similar role in B cells, coupling to at least PLC $\gamma$  and Grb2 (186) (Fig. 4). Both T-cell and B-cell Ag receptor engagement leads to activation of the Ras/ERK pathway (through Grb2 and Sos, and through PKC activation), PI3K (through p85), PKC (through PLC $\gamma$  and DAG), and the phosphatase calcineurin (through PLC $\gamma$ , IP3, and Ca<sup>2+</sup>). Recruitment and activation of PI3K (and perhaps other key signaling molecules) can be augmented by binding sites formed on costimulatory receptors that augment responses to antigen receptor engagement (CD19 in B cells; CD28 in T cells) (176).

Another adaptor protein, SLP-76, becomes phosphorylated in T cells and associates with LATS through another adaptor, Gads. SLP-76 recruits Vav, which becomes TYR phosphorylated and activated (augmented by simultaneous stimulation of the CD28 coreceptor). Vav is a GTP exchange factor for Rac activation (187), which can lead to cytoskeletal changes and activation of the p38 and JNK stress signaling

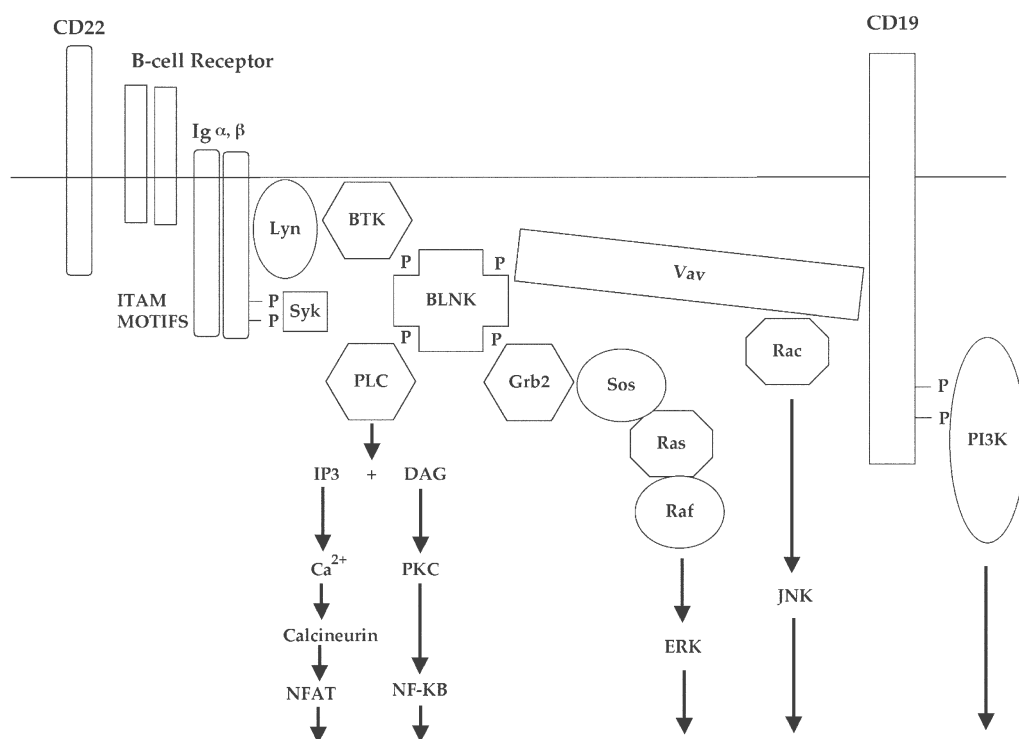


Fig. 4. BCR signaling. Ag stimulation leads to dephosphorylation and activation of Lyn. This results in tyrosine phosphorylation of receptor-associated Igα- and Igβ- subunits on ITAM motifs. This leads to recruitment and activation of further tyrosine kinases, Syk and Btk, followed by phosphorylation of an adapter protein, BLNK. Phosphorylated BLNK recruits PLC, Grb2 (leading to Ras activation), and Vav. Facilitated by stimulation of the coreceptor CD19, Vav activates Rac and hence JNK pathway activation. CD19 also can recruit and activate PI3K. The net consequence of receptor stimulation is activation of Ras/ERK, PI3K, and PLC pathways as for RTKs. Here, downstream activation of NF-κB and NFAT proteins is prominent in supplementing the outputs diagrammed in Figs. 1 and 2.

MAPK pathways not generally activated by nonhematopoietic growth factors acting directly on RTK.

In addition to targets of Ras/ERK and PI3K pathways, Ag receptor pathways activate the transcription factors NFAT, by calcineurin-mediated dephosphorylation, and NF-κB, through PKC activation of IKK (117). NFAT activity is especially important for instructing expression of suitable cytokines for immune cell interactions (178). NF-κB shares this role but additionally provides crucial antiapoptotic signals (e.g., by inducing Bcl-2 and Bcl-xL) to supplement those of Ras/ERK and PI3K pathways (117,118).

B and T cells undergo chromosome rearrangements at various stages of their development to express appropriate BCR and TCR complexes. They are especially prone to mutations involving aberrant chromosome rearrangements, including translocations.

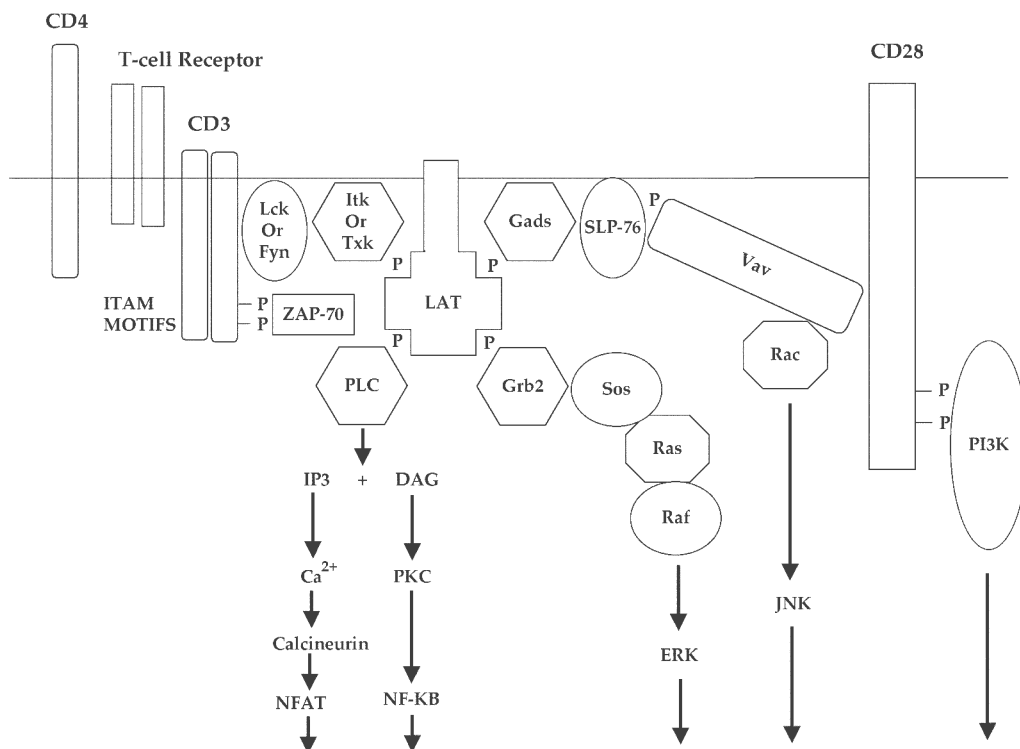


Fig. 5. TCR signaling. Stimulation of the TCR with major histocompatibility complex-bound peptide elicits a very similar response to that described in Fig. 4 for B cells. The identities of the adapter proteins, receptors, and coreceptors and the precise members of tyrosine kinase families involved are different, but the basic principle of activating three classes of tyrosine kinases, followed by recruitment of adapters and effectors, is the same.

Several lymphoid cancers originate from translocations juxtaposing protooncogenes and active enhancers such as IgH enhancers in B cells. Prominent examples include activation of Bcl2 in follicular lymphomas, cyclin D1 in mantle cell lymphomas, and c-Myc in Burkitt's lymphoma (188). These genetic alterations affect cell cycle, cell growth, and apoptosis. In addition, several lymphomas are associated with activation of NF-κB target genes, and in some cases with underlying mutations in NF-κB or I-κB genes, emphasizing the importance of this pathway in lymphoid cells for regulating apoptosis and (through Myc induction) cell proliferation. Another class of mutations found specifically in lymphomas involves Bcl6, which regulates aspects of cell cycle as well as the activity of a regulator of B-cell differentiation, Blimp. Presumably such mutations prevent differentiation and retain cells in a state where they can be induced to proliferate.

#### Information Content of Tyrosine Kinase Signaling Pathways

The preceding discussions of RTK pathways, integrin pathways, GPCR pathways, Ag receptor pathways, and cytokine pathways show that a large number of ligands that influence proliferation connect to a restricted group of signaling pathways involving

TYR kinase phosphorylation at an early step and then activate Ras, PI3K, and, more variably, a small number of other key signaling molecules such as PLC,  $\text{Ca}^{2+}$ , and Rac/Rho family GTPases. The widespread use of Ras-centered pathways to regulate growth (among other events) means that most cell types contain the signal-transduction machinery that could translate mutational activation of these signaling pathways into a proliferative response. This finding is consistent with association of activated Ras with a variety of tumor types. However, activation of these same pathways in normal development and physiology can lead to a variety of outcomes other than growth. It is therefore important to ask whether the extent of activation of a given pathway or the relative balance of different pathways is instrumental in determining the cellular response.

#### QUANTITATIVE ASPECTS OF RTK SIGNALING

Some tissue culture models suggest that quantitative aspects of the Ras/ERK pathway determine whether Ras/ERK activation is interpreted as a proliferation signal or not. The pheochromocytoma cell line PC12 can be induced to proliferate by activation of EGFRs or to differentiate by stimulation of NGFRs (189). Each RTK activates a variety of signaling pathways, but introduction of activated MEK alone can induce differentiation in PC12 cells and proliferation of fibroblasts, suggesting that the Ras/ERK pathway might suffice to specify the response, perhaps dependent on the quality of the signal (189). EGFR is more rapidly internalized and downregulated than NGFR in PC12 cells and leads to a more short-lived stimulation of activated Ras. If EGFR is first overexpressed, then EGF can elicit PC12 cell differentiation. Conversely, variant PC12 cells selected to proliferate in response to NGF were found to have reduced numbers of NGFRs. These results were interpreted as defining the duration of Ras/ERK signaling as critical, drawing attention to the observation that only sustained activation leads to discernible nuclear accumulation of ERK (189). It is difficult to separate the contributions of the duration of signaling from those of signal magnitude, and it is very likely that in all cases of Ras/ERK signaling at least some ERK reaches the nucleus to act on transcription factors and elicit a response.

Studies using conditionally expressed activated versions of the Raf family in NIH3T3 cells re-examined issues of Ras/ERK signal strength and duration. In this case, high levels of Ras/ERK signaling were found to promote quiescence in preference to proliferation (190,191). For example, overexpressed activated A-Raf induced smaller changes in ERK activity than similarly expressed Raf1 or B-Raf but was unique in stimulating proliferation. Conversely, a hyperactivated form of A-raf, created by introducing mutations, was a more potent activator of ERK than the parent protein but failed to induce proliferation. Excessive Raf activity made the NIH3T3 cells refractory to PDGF growth stimulation. In all of these situations, including stimulation by extracellular mitogens, strong stimulation of the Ras/ERK pathway induced the CDKI p21, and p21 induction correlated with cell-cycle arrest (51,190–193). Similar correlations have been observed in hepatocytes as well as fibroblasts, suggesting that there may be a general connection between high levels of Ras/ERK signaling and p21 induction (51).

Induction of cyclin D1 by a variety of mitogens or by expression of Ras/ERK pathway components required only low levels of ERK activity. It was generally found that the signal must persist for several hours (51). One possible mechanism that could explain a requirement for sustained signaling is synergy between transcriptional induction and protein stabilization of ERK targets (194,195). For example, *fos* mRNA is rapidly induced by ERK signaling, but Fos protein is rapidly turned over in the absence

of phosphorylation and stabilization by RSK (itself activated by ERK) and ERK (196,197). Only during sustained ERK signaling would Fos protein accumulate significantly. Other ERK targets (such as Myc) that are regulated transcriptionally and by protein stabilization may behave similarly. Effective induction of these protein activities would require sustained ERK activation in contrast to targets induced only by transcription. The spectrum of genes induced by ERK activation changes over time from those regulated acutely (e.g., by Ets protein phosphorylation) to those regulated by intermediate early gene products like Myc and the various Fos/Jun dimers constituting AP-1 activities. Thus, for cell proliferation, plausible mechanisms corroborate the empirical observations that both the intensity and duration of Ras/ERK signaling can influence outcome. The outcome of RTK signaling is, of course, also greatly influenced by pathways other than Ras/ERK that are simultaneously activated, especially the PI3K pathway. Stimulation of proliferation and cell transformation by activated Ras is in some cases dependent on all of these pathways, and not just the quality of Ras/ERK signaling (52,198).

#### CELLULAR RESPONSIVENESS; DEVELOPMENTAL HISTORY

Even though signal strength, duration, and diversity can affect the outcome of RTK pathways, it is clear that the quality of pathway activity is not sufficient to define the cellular response (199). The developmental history of a cell is crucial. For example, in the developing *Drosophila* eye, the response to EGFR and Sevenless RTK in inducing specific transcriptional targets and cell fates is determined either by the presence of specific ancillary transcription factors or by exposure to additional signaling pathways (199–201).

In some situations, the function of an RTK signal may be binary (ON or OFF: corresponding perhaps to the presence or absence of a neighboring signaling cell) and the cellular response preprogramed by other factors. Activation of the EGFR in the *Drosophila* eye translates to many cell behaviors: differentiation as an outer photoreceptor cell, differentiation as an inner R7 photoreceptor, cell division, or cell survival, depending on each cell's history. Cells that are not close to a cell secreting an EGFR ligand at these different stages fail to either differentiate, divide, or survive. At any one time the choice is between two fates, and a third fate cannot be imposed artificially by manipulating the extent of RTK pathway activation.

This concept is important when considering other signaling pathways. In essence all are potentially generic. They can be used to regulate cell fate, differentiation, proliferation, or cell death, with the outcome dependent on the prior history of a cell. The evidence for this is indisputable; that is, examples can be found for each type of outcome for nearly every signaling pathway that has been investigated. While growth factor–signaling pathways were first studied in connection with cell proliferation, they can affect all aspects of cell behavior, including cell-fate determination and differentiation. Several pathways that were studied initially as developmental pathways influencing cell fate are now also known to affect cell survival and proliferation, depending on the cellular context. All signaling pathways have the potential to affect the cardinal behaviors associated with cancer development; therefore, we cannot always expect to associate a certain pathway with a specific cellular response. Nevertheless, we can look for patterns and generalizations. During normal development, where the emphasis is on cell-fate determination, very few generalizations can be made. Every major signaling pathway contributes to cell-fate assignments. When dealing with cancer development,

there is some pattern. TYR kinase pathways often appear to be involved at mid-stages of cancer development in promoting proliferation, and TGF- $\beta$  pathways are less frequently involved and generally inhibit proliferation, whereas Wnt and Hedgehog pathways regulate the earliest steps in cancers of specific cell types.

### *TGF- $\beta$ Family Signaling*

Although TGF- $\beta$  was first defined as an activity stimulating cell proliferation in culture (202), it is clear that the TGF- $\beta$  superfamily is composed of two major branches TGF- $\beta$ s (or TGF- $\beta$  proteins) and bone morphogenetic proteins (BMPs) and that these ligands elicit a huge variety of cellular responses in a variety of organisms (203–206). Members of the TGF- $\beta$  family can stimulate proliferation of some cells in culture but more often exert an inhibitory role that can prevent growth, even of some tumor-derived cells (207). The BMP subfamily in particular has many roles in vertebrate and invertebrate development that have a number of interesting characteristics, including dose-dependent responses, which allow them to act as morphogens, instructing cell fate according to spatial concentration gradients (208).

#### SIGNALING PATHWAY

All TGF- $\beta$  family proteins are active as dimers, but heterodimeric partnerships are permissible, including association with ligands that form an inactive complex. The expression of BMP is highly regulated, but in many cases, production of inhibitory ligands is a key spatially restricted, developmental event (209–211). The Spemann organizer (the most dorsal and earliest invaginating mesoderm of *Xenopus*) produces molecules, such as Chordin and Noggin, that bind to BMP4 and inhibit induction of epidermal cells, leading to adoption of the default neural fate. Furthermore, extracellular Chordin and related molecules can be cleaved by specific proteases whether alone or in complex with BMP, modifying the spatial distribution of Chordin and providing a means to transport inactive complexed BMP before activation by Chordin proteolysis at a distant site (212,213). TGF- $\beta$  family molecules are antagonized extracellularly by binding to Follistatin, Inhibin, Lefty, and DAN/Gremlin/Cerberus family members in addition to Chordin and Noggin (209,210).

Two types of receptor SER THR kinases (types I and II) are required to respond to TGF- $\beta$  family ligands. Initial binding of ligand to the type II receptor (sometimes enhanced by an ancillary glypican receptor such as Endoglin, Crypto, or Betaglycan) recruits type I receptor, which also binds to the ligand (210). The type II receptor is constitutively active and phosphorylates the type I receptor within a ligand–receptor complex that leads to its activation (206,214,215). Mutationally activated type I receptor is sufficient to propagate the signal. Even though both types of receptor contribute to ligand recognition, the specificity of the intracellular response is dictated solely by the type I receptor. Furthermore, the seven or more vertebrate type I receptors only transmit two types of signal to their key targets, Smad proteins. The many members of the TGF- $\beta$ /activin family of ligands activate two specific receptor-regulated Smads (R-Smads), Smad2 and Smad3, whereas all BMP family ligands activate the R-Smads, Smad1, 5, and 8.

The receptor-regulated subfamily of Smad has a conserved N-terminal MH1 domain and a conserved C-terminal MH2 domain, which interact and are inert before phosphorylation of the C-terminus by the activated type I receptor. This activation step promotes heterodimerization with a second type of Smad protein, Smad4 (which does not



interact directly with receptors), and allows entry of the Smad complex into the nucleus. Both events are essential to elicit a transcriptional response (216). In most cases, the Smad complex associates with another DNA-binding protein (e.g., FAST-1 for a specific functional site on the activin-responsive *Mix-1* gene promoter); in other cases, the low-affinity DNA-binding activity of the MH1 domain suffices to target the complex to important promoter regulatory elements (217). In each case, the MH2 domains of the Smads can provide transcription activation function. The Smad DNA-binding partners add further DNA-binding specificity to influence which target genes are affected by signaling. They may also bring transcriptional activation domains, or coactivators or corepressors, regulating to what extent genes are activated or repressed by TGF- $\beta$  signaling. Those partners can be expressed differentially according to cell type and may themselves be subject to regulation by other signaling pathways, as exemplified by Jun and LEF/TCF proteins (effectors of Wnt signaling; see below). A cell's history and exposure to additional signals greatly affect its response to TGF- $\beta$  ligands.

Inhibitory Smads often are transcriptionally induced by TGF- $\beta$  family signaling. These Smads can act by competing with receptor-regulated Smads for association with type I receptor or by inhibiting heterodimerization of activated Smads with Smad4 (218,219).

Although Smads are the only clearly defined mediators of TGF- $\beta$  signals, it is commonly observed that various MAPK pathways can be activated by TGF- $\beta$  ligands without Smad participation (215,220). While TAK1 and Rho family proteins have been implicated as intermediates, there is no clearly understood mechanism for this. On the other hand, specific ERK phosphorylation sites have been identified on Smads that can inhibit transcriptional responses to TGF- $\beta$  family ligands, providing a mechanism for regulation of TGF- $\beta$  signaling by MAPK pathways (219,221–223).

Overall, the major signal transduction route for TGF- $\beta$  family members is very simple compared to the network of changes elicited by RTK activation. The pathway responds to a large number of ligands that are subject to extensive extracellular regulation, pathway activity can accurately reflect ligand dose, and it can produce diverse responses according to the specific transcriptional cofactor associated with Smads in the nucleus.

#### CONNECTIONS OF TGF- $\beta$ PATHWAYS TO CELL PROLIFERATION

TGF- $\beta$  proteins regulate many cell-fate choices during development, but also have direct effects on apoptosis and cell proliferation (203,204). TGF- $\beta$  ligands frequently promote apoptosis, e.g., to eliminate webbing between limb digits (224–227). TGF- $\beta$  ligands most commonly limit cell proliferation, especially of hematopoietic and lymphoid cells, but they can also stimulate proliferation of some cells, notably germline stem cells (228,229).

The most prominent mechanisms by which TGF- $\beta$  ligands are known to inhibit cell proliferation involve induction of CDKIs and repression of Myc (206). Both p15INK4b and p21 are induced by TGF- $\beta$  in a variety of cell types. This induction requires the association of activated Smad with an unknown DNA-binding partner and recruitment of this complex to the p15 promoter by Miz-1, a protein that interacts with the initiator region (230). In the absence of TGF- $\beta$  signaling Miz-1 recruits Myc, leading, unusually for Myc, to direct repression of p15. Hence, p15 activation also requires TGF- $\beta$  signaling to limit this repressive Myc function, which is accomplished by formation of a repressive Smad/E2F4/5/p107 repressor complex at the Myc promoter, thereby reducing *myc* transcription (231).

MUTATIONAL ALTERATION OF TGF- $\beta$  PATHWAYS IN CANCER

Several types of mutations affecting TGF- $\beta$  signaling have been implicated in carcinogenesis (206,232–234). The most frequent mutation associated with human tumors is loss of Smad4 function, which appears to have an essential role as a partner for all receptor-activated Smads and the growth inhibitory role of TGF- $\beta$  signaling. Loss of heterozygosity for the genomic region, including Smad4, is seen very frequently in human pancreatic carcinomas (approx 50%) and in colorectal tumors (approx 50%) (206). In the intestinal crypts, TGF- $\beta$  I and TGF- $\beta$  II are expressed in cells near the lumen, implying a possible role in slowing proliferation and inducing differentiation as cells move and mature from the base of the crypts toward the lumen. TGF- $\beta$  type II receptor mutations have been found in tumors, especially when genomic instability was induced by the absence of DNA repair enzymes, as in hereditary nonpolyposis colorectal cancer (235,236). TGF- $\beta$  type I receptors are mutated in several cancers of different origin (206).

Mouse models confirm the antitumorigenic roles of TGF- $\beta$  signaling. Mice lacking Smad3, an effector for TGF- $\beta$ 1, develop lethal colorectal adenocarcinomas before 6 months, implying that failure of TGF- $\beta$  signaling can suffice to promote tumor formation (237). Mice that are heterozygous for Smad4 (homozygotes die early) develop polyps and late-onset tumors at an enhanced rate, and can markedly exacerbate the progression of polyps initiated by heterozygosity for the tumor suppressor adenomatous polyposis coli (APC) (238,239).

Despite the thoroughly documented involvement of TGF- $\beta$  pathways as tumor suppressors in humans and mouse models, TGF- $\beta$  signaling probably facilitates late stages of cancer development (206,234). These oncogenic effects are likely to involve cell migration and invasiveness but have not been dissected in detail (206,240).

*Wnt Signaling*

The Wnt name stems from the realization that *Drosophila* Wingless, which affects many developmental decisions, and *int-1*, which can induce tumors if overexpressed in response to insertion of a retrovirus, were similar in sequence and action (241). The mechanisms of Wnt signaling have been studied largely in a developmental context in mice, zebrafish, *Drosophila*, *Caenorhabditis elegans*, and *Xenopus* (241,242). As for TGF- $\beta$  family members, Wnt proteins can act as morphogens, indicating dose-dependent signal transduction (208). Their activity can be regulated not only by controlling their expression and movement between cells but also through the production of secreted homologs of Wnt receptors and other molecules that can bind Wnts and inhibit their actions (243). Other extracellular proteins can inhibit Wnt actions by competing for a Wnt coreceptor (244), and glycoproteins are required for Wnts to interact productively with receptors (245). All of these features plus the existence of several Wnt family members confirm the extensive use of Wnts during development and the need to control their distribution and activity very carefully.

## SIGNALING PATHWAY

The primary receptors for Wnts are transmembrane proteins of the Frizzled (Fz) family. Most initial studies on Wnt signaling and the actions most relevant to cancer involve alterations in gene expression through the Wnt/ $\beta$ -catenin pathway. In this pathway, LRP5 or 6 low-density lipoprotein receptor-related protein family members act as essential coreceptors (246). Other Fz-mediated pathways, collectively

referred to as noncanonical, include those that act through phospholipids and calcium (the Wnt/Ca<sup>2+</sup> pathway), or through Rho family GTPases and other means (planar cell polarity pathway), and do not involve LRP5/6 (247–250).

Fz receptors have seven transmembrane domains, characteristic of G-protein-coupled receptors, and it appears that the Wnt/Ca<sup>2+</sup> pathway is activated through a G-protein, leading to activation of PLC, generation of DAG and Ca<sup>2+</sup>, and activation of PKC and calcium-calmodulin-dependent protein kinases (248,251). Although Fzs activated in the Wnt/β-catenin pathway can also activate G-proteins, the relevance of this to pathway activity is quite uncertain (249,252).

The central regulatory step in the Wnt/β-catenin signaling pathway is the regulation of ubiquitin-mediated proteolysis of β-catenin (241). In epithelial cells, the bulk of β-catenin normally associates with the homophilic, calcium-binding transmembrane adhesion molecule, cadherin, and with α-catenin, which can bind actin. This adhesion complex links the actin cytoskeletons of apposed cells and is required for maintaining the epithelium. When β-catenin is present in excess of cadherins, it is rapidly degraded by the ubiquitin proteolysis pathway (249). Degradation is triggered by multisite phosphorylation of the N-terminal region of β-catenin initiated by casein kinase 1 and continued by GSK3 (253). Degradation of β-catenin can be inhibited by mutational alteration of the key phosphorylation sites or by inhibiting the activity of GSK-3 or CK1. Wnt signaling also reduces degradation of β-catenin by inhibiting phosphorylation, but the precise mechanism for this action, is not clear (249).

The regulation of β-catenin phosphorylation involves several additional players. Axin can bind GSK-3, CK1, and β-catenin and promotes phosphorylation of β-catenin (254,255). APC-protein can bind to both axin and β-catenin, especially after it is phosphorylated by GSK-3, stabilizing the complex and further favoring β-catenin phosphorylation (256,257). These interactions are seen in a variety of cell types and organisms and are essential to keep the pathway silent in the absence of a Wnt signal. One clear mechanism by which Wnts can disrupt the cycle of β-catenin phosphorylation and degradation has been described, and it may not operate universally. This involves another GSK-3-binding protein (GBP), which competes with axin for GSK-3 binding (249,258,259). GBP may be brought to the β-catenin complex by the Disheveled (Dvl) protein. This action of Dvl is thought to be triggered by Fz receptor activation, but whether Dvl interacts directly with the Fz receptor is not clear. Axin can bind to the LRP5/6 coreceptor and this may serve to bring the β-catenin complex together with receptors and Dvl (246).

Stabilized cytoplasmic β-catenin can move to the nucleus and associate with Tcf/Lef family transcription factors (260). Tcf/Lef proteins bind to DNA but cannot activate transcription alone; β-catenin provides a strong transcription activation domain at its C-terminus. The β-catenin/Tcf complex can bind specific sites on DNA and stimulate transcription (261). In the absence of β-catenin, TCF proteins associate with Groucho family proteins to repress transcription. Wnt signaling can derepress as well as activate target genes with Tcf-binding sites (260). Perhaps for this reason, there is considerable evidence that in some circumstances β-catenin need not enter the nucleus and activate transcription, and that it may instead act primarily to remove Tcf repressor from engagement with DNA (262,263).

#### CONNECTIONS OF WNT PATHWAYS TO CELL PROLIFERATION

Wnt/β-catenin signaling can affect the expression of large numbers of genes but two targets, Myc and cyclin D1, have drawn particular attention as potential mediators of

proliferative responses to Wnts (264,265). Wnts do commonly stimulate proliferation of cells during development (111,266–268), but, as for other pathways, it is also possible to find circumstances in which Wnts contribute to arrest of cellular proliferation instead (269).

#### MUTATIONAL ALTERATION OF WNT PATHWAYS IN CANCER

Mutations in APC in colon carcinomas provide the most dramatic example of Wnt-pathway mutations in cancer (270,271). Such mutations are found not only in heterozygous form in the germline of individuals with predisposition to multiple colon polyps and cancer but also in most spontaneous colon carcinomas, generally as truncation mutations accompanied by complete loss of the second allele. In such colon carcinomas and in melanomas,  $\beta$ -catenin is stabilized when APC mutations are present, and in many of the cancers that retain APC function, mutations affecting the N-terminus of  $\beta$ -catenin are found. This suggests that stabilization of  $\beta$ -catenin and activation of Wnt/ $\beta$ -catenin pathway target genes is key to the tumor-promoting activity of APC mutations. This finding does not exclude the potential importance of additional consequences of APC mutations, which might affect cell migration and survival independent of effects on  $\beta$ -catenin (271). Since APC mutations are found in the earliest stages of colon cancer, it is thought likely that such mutations initiate tumor development, and the APC gene has been termed a gatekeeper for this tissue (272,273). Mouse models in which the phosphorylated region of  $\beta$ -catenin has been removed also lead to adenomatous polyps in the intestine (rather than the colon), emphasizing this gatekeeper role (271,274). Clearly, there is a strong association between APC and protecting the epithelium of the intestine from cancer, but there is ample evidence from human tumors and mouse models that the Wnt/ $\beta$ -catenin pathway can participate in the development of tumors at other sites, especially in melanomas and hair-follicle tumors (270,275).

The normal development and maintenance of hair follicles and the intestinal epithelium has been fairly well studied (276–278). In each case, Tcf activity and Wnt/ $\beta$ -catenin signaling have been implicated in the normal processes of stem cell maintenance or differentiation, leading to the idea that stem cells in these tissues may be the specific target of oncogenic mutations affecting the Wnt/ $\beta$ -catenin pathway (260,279–283).

#### Hedgehog Signaling Pathways

The Hedgehog (Hh) family of proteins was first uncovered in *Drosophila*, where its principal role is to control cell fate by inducing changes in gene transcription (284). Hh signaling can lead to cell proliferation, in some cases directly, and has a role in survival of some cell types. Vertebrate Hh proteins (Sonic, Desert, and Indian Hedgehogs in humans and mice) control many aspects of development, including patterning of the neural tube, somites, and limbs (285,286). Excessive or inappropriate Hh signaling can produce tumors and, in humans, is associated principally with basal cell carcinoma (BCC) and less frequently with medulloblastomas and other cancers. Hh-signal transduction involves the relief of multiple inhibitory constraints on the activity of transcriptional activators of the GLI family of zinc-finger DNA-binding proteins (originally identified as being amplified in gliomas). The signaling pathway is best understood in *Drosophila*, where the GLI homolog is called Cubitus interruptus (Ci), but most of the interactions revealed in *Drosophila* have been found to be applicable to vertebrates as well.

## SIGNALING PATHWAY

Binding of Hh to its receptor, Patched (Ptc), releases an inhibitory constraint on the seven-pass transmembrane protein, Smoothed (Smo). The mechanism for this was originally thought to involve an allosteric change or complete disruption of Ptc:Smo complexes but is now thought unlikely (249,287–289). Unproven, postulated mechanisms currently involve regulated localization of Smo within the cell as well as regulation of Smo activity by low-molecular-weight compounds (presently identified) that might be pumped across membranes by Ptc.

Smo is similar in structure to Fz proteins and, as for Fz, it is unclear whether it activates G-proteins (249). Somehow Smo activation leads to multiple regulatory events that affect Ci activity. In the absence of Hh, the primary Ci translation product (Ci-155) forms complexes with various proteins (including Costal-2, Fused, and Suppressor of Fused); binds to microtubules; and undergoes partial proteolysis that produces a relatively stable product, Ci-75. Ci-75 has the same DNA-binding specificity as Ci-155 but acts as a transcriptional repressor (249). Although processing of Ci-155 to Ci-75 is slow (several minutes to hours) in the absence of Hh, the activity of Ci-155 is held in check by associated proteins, restricting access to the nucleus. Hh signaling inhibits proteolysis of Ci-155 to Ci-75; frees Ci-155 from microtubules; and facilitates the conversion of Ci-155 into a transcriptional activator, stimulating accumulation of at least a small proportion of Ci-155 in the nucleus. Hh signaling causes derepression by eliminating Ci-75 and activation of the same target genes through Ci-155, a principle similar to that used in Wnt signaling. How Smo activation accomplishes these feats is unknown, but the process undoubtedly involves regulated protein phosphorylation.

The proteolysis of Ci-155 requires phosphorylation of Ci at PKA sites. Once phosphorylated at these sites, Ci can be further phosphorylated by GSK-3 and CK1 (249). Each of these phosphorylation events is required for proteolysis to Ci-75. As for Wnt signaling, the way in which Hh regulates phosphorylation by GSK-3 or CK1, or acts in other ways to stabilize Ci-155, is not clear. It is possible that Hh signaling acts to disrupt a Ci complex that facilitates phosphorylation, but this has not been shown. Most of these signaling interactions are already known to be conserved in vertebrates, but there are also some clear differences. The activities of Ci are distributed among three GLI proteins (286). GLI3 acts largely as a transcriptional repressor that is activated by proteolysis that can be inhibited by Hh signaling; GLI2 acts largely as an activator that may be regulated by proteolysis (not necessarily controlled by Hh); and GLI1 acts as an activator that is frequently regulated transcriptionally in response to Hh signaling activity, thereby amplifying and maintaining an initial response.

## CONNECTIONS OF HH PATHWAYS TO CELL PROLIFERATION

Direct effects of Hh signaling on cell proliferation have not been studied as extensively as RTK pathways, so many intermediates remain to be discovered. So far *Myc*, cyclin D, and cyclin E genes have been identified as significant targets that are transcriptionally induced by Hh signaling in vertebrates or in *Drosophila* (290–292).

## MUTATIONAL ALTERATION OF HH SIGNALING PATHWAYS IN CANCER

Loss of function mutations in Ptc, activating mutations in Smo, and overexpression of GLI proteins have been associated with human cancers (286,293–295). As for Wnt signaling, there is a marked specificity for the target tissue. BCC inevitably arises in individuals heterozygous for a *ptc* mutation, and activation of the Hh pathway is

almost invariably associated with sporadic BCC. Overexpression of Hh from a keratin promoter in mice leads to very rapid and widespread development of tumors similar to BCC (296). These observations suggest that activation of the Hh pathway is a very early, obligatory step in development of BCC. Less persuasive or comprehensive evidence and arguments link Hh signaling to a small number of other cancers, including medulloblastomas (293,295,297). Tissues susceptible to Hh pathway-associated carcinogenesis rely on Hh signaling for normal cell proliferation, and it is plausible (though far from proven) that this may be due to effects of Hh signaling on stem cells (282).

## Development of Cancers

### *The End Point: Cancer Cell Traits and Their Interdependence*

This chapter has discussed some of the essential properties of cancer cells (growth, cell cycling, and survival independent of environmental signals) but has ignored other equally important properties (telomere maintenance, promotion of angiogenesis, tissue invasion, and metastasis) (130,150), because knowledge of their links to growth factor–signaling pathways is fairly limited. Undoubtedly, there are extensive links. An important regulator of angiogenesis, vascular EGF (VEGF), is induced by several signaling pathways, as are the protein component of telomerase (induced by Myc) and several proteases that could facilitate tissue invasion (298–301). Likewise, cell movements, exit from epithelia, and migration between cells have all been linked to specific signaling pathways during normal development in a variety of model organisms and in cell culture (302). Despite these various insights, the association of specific mutations with acquisition of these behaviors in cancer cells is not apparent in the same way as Ras, Myc, RB, and p53 have been associated with cell proliferation and survival. This, and the sufficiency of some of these mutations for inducing tumors rapidly in mouse models, has led some people to suggest that acquisition of proliferative and survival properties represents the only critical rate-limiting step in cancer, from which all other aspects of cancer development follow inexorably (114). In other words, once a group of cells guarantees its survival and proliferation, it will assemble into a viable tissue, much as normal cells organize themselves, and because those cells do not rely on specific local environmental signals, they will be able to grow in other locations.

It seems reasonable to consider invasion and metastasis as properties of cancer cells that must inevitably follow the initial development of cancerous cells at one location. It is not clear to what extent cell proliferation, cell survival, angiogenesis, telomere maintenance, and genomic instability are independent, sequential attributes adopted by cancer cells. We have seen that mutational disruption of cell-cycle controls often induces apoptosis, so it can be argued that antiapoptotic pathways must first be activated for a cancer cell to develop. Likewise, it could be argued that telomere loss will limit the lifespan of a cell before it can acquire the several mutations required to become a cancer cell. Telomerase induction or an equivalent protection must therefore be induced relatively early in cancer development (or the target cell must start with high telomerase activity, as many stem cells do) (130). Similarly, mutations affecting genomic stability must occur relatively early if they are to have any impact on the rate of accumulation of significant mutations (303). Before cancers become large, angiogenesis will be required to provide oxygen and nutrients for cell growth and to avoid cell death.

Selection must play a huge role in the development of a cancer. Many cell divisions are required to accumulate a large number of mutations, and many cells must die as a consequence of lethal mutations. Most important, the susceptibility of cells with muta-

tions affecting the cell cycle imposes a selection for mutations affecting apoptosis. Mutations affecting genomic stability impose a selection for mutations that inactivate DNA damage checkpoint controls. Mutations that produce cell proliferation impose a selection for angiogenesis. We can deduce how certain mutations encourage the more rapid emergence of other traits (even against a constant rate of induction of random mutations), and how certain traits may be the first to be selected. For example, mutations conferring proliferation to normally relatively quiescent cells would increase the proportions of clonal derivatives of such cells, as would promoting the survival of cells that normally have a short life (such as derivatives of stem cells in intestinal epithelium crypts). Selection for genomic instability is not easily rationalized, so it has been suggested that in some cases this trait may be carried along by coselection of another type of mutation in the same cell (304). This selection could be relatively frequent if there are many potential targets that can induce genomic instability as a consequence of a single mutational hit. Alternatively, one could argue that once there is selective pressure for a new mutational trait (e.g., inactivation of p53 or the equivalent), then this is directly favored by an intermediate step in which genomic instability is induced. These types of ideas set the background for how cancers might develop. They are only hypotheses, for what is required is direct observation of cancer development.

### ***Progress Toward the End Point: Inferences From Normal Development***

Unfortunately, only limited opportunities are available to observe cancer development. Correlation of stages of human cancers with acquisition of specific mutations has only been accomplished on a large scale for colorectal cancers (305–307). Mouse models provide an easier opportunity to study stages of development of specific tumors that are induced at high frequency by targeted mutations or transgenes (308,309). Even this approach is not straightforward, and clear differences between the effects of specific mutations in mice and humans are apparent (150,309).

Normal cells behave according to their history and environment. Key behaviors include cell growth and division, cell death, mitotic arrest, differentiation, cell movement, signaling to other cells, and altered sensitivity to environmental signals. These behaviors can be explained with ever-improving clarity in terms of molecular changes: changes in gene transcription, RNA processing, protein translation, modification and degradation, physical molecular interactions, and movements of molecules within the cell. In particular, many studies describe the molecular steps by which exposure to a single new environmental signal leads to specific changes in cell behavior. It is generally believed that by identifying when cells are exposed to new signals and understanding the consequence of each such signaling episode, one can understand how cells collaborate to choreograph the changes in cell behaviors that constitute development of a multicellular organism. That objective is very challenging because of the large number of cellular and molecular interactions involved, but it is facilitated by the reproducibility of development among individuals of a species and the potential to observe changes in cells as they take place, at least in model organisms. Systems of study are already in place that guarantee rapid progress in understanding normal development and that allow fairly rigorous testing of hypotheses. Normal development already is, and will continue to be, a source of robust examples of how cells behave according to extracellular signals.

In human cancer, cells still behave according to history and environment, but the acquisition of mutations becomes an important part of a cell's history, and, ultimately,

the environment is formed by the cancer cells themselves rather than by surrounding normal tissue. Many of the significant mutations in cancer cells seem to cause specific changes in cell behavior, such as promoting proliferation or cell survival, either because they mimic normal environmental signals or because they alter the molecules that more directly control cell behavior. However, very few situations exist in which the predicted consequence of a specific mutation can be observed directly. In addition, the answers to several important questions generally are not known: Which are the precise cells that have the first mutations on the path to cancer? What is the sequence of acquired mutations? How do the developing cancer cells interact with their normal environment and create their own environment? These questions will have different answers for different cancers, just as for different cell types during normal development. Even for one type of cancer, however, one generally cannot observe the course of development, nor can one expect that the development of a particular type of cancer will necessarily be reproducible. Mouse models can certainly help the study of cancer development, as can the more limited studies possible of the progression of human cancers. Presently no satisfactory solution exists for this problem. If hypotheses are made based on knowledge of normal development, this is likely to be most informative for the earliest stages of cancer when cell behaviors are still largely constrained by their normal environment. The key themes from normal development that need to be considered carefully with respect to cancer development are the impact of a cell's history on its molecular response to a new signal or mutation, the potential for cells to collaborate in building a cancer as for tissue formation, and the necessity to identify the cell of origin and the various cell types deriving from that cell during formation of a cancer.

### *Cell History: Signaling Episodes and Altered Responsiveness*

During normal development, complex patterns of cell fate, proliferation, and cell death build from simple beginnings. At the very earliest stages, two daughter cells of a fertilized egg sometimes become different from each other by asymmetric partitioning of key regulatory molecules. At this stage, the daughter cells sometimes have different contacts with maternal or maternally deposited cells or molecules that can instruct differences in subsequent behavior by a signaling mechanism. The differences in the two daughter cells (instilled by either mechanism) can lead *inter alia* to differential production of signaling molecules and differences in response to a given signaling molecule. A more complex signaling environment is built up as more cells are produced and induced by increasingly local environments to produce new, and different, signals. The cells also acquire different sensitivities to signals according to their history. Hence, a relatively small set of signals can induce cells to adopt an increasingly varied set of behaviors, governed essentially by their position and heritage. Position determines exposure to environmental signals, whereas a cell's heritage is manifest as patterns of gene expression, disposition, and modifications of various macromolecules, especially proteins involved directly in signal transduction and transcription factors that influence which genes are regulated by signal transduction pathways.

This model can generate diversity but cellular diversity must be precisely organized. If all cell behaviors were continually in a state of flux according to changing signals and responsiveness, it would likely be very difficult to integrate a series of cellular interactions into a developmental plan that is extremely reproducible and resilient to stresses and errors. It is more attractive to imagine that signaling episodes are clearly punctuated and that discrete yes/no decisions are made as a result of each type of inter-



action (i.e., proliferate or not, turn on a specific gene or not). This idea acknowledges that quantitative levels of signaling can be crucial, that signaling episodes can have very different frequencies, and that a specific cell can be influenced by a long-term signal while responding to a different signal of shorter duration. It does require that cells can sometimes remember a decision indefinitely and can terminate their response to a specific signal, so that exposure to a new signal, or even the same signal, can subsequently produce a different molecular response. Some clear examples of these features can be found in development. In *Drosophila* embryos, the patterns of expression of homeotic genes are first set up according to interactions of multiple transcription factors, but after this period of establishment, cells continue to express a specific homeotic gene (or two) without continued input from the proteins that initiated this expression (310). Instead, the expression pattern is cemented by a stable, heritable organization of chromatin. The proteins that organize and maintain expression patterns through chromatin organization are present in vertebrates, and it is likely that similar mechanisms of remembering certain decisions are used.

Studies in the *Drosophila* eye have provided striking examples of the episodic nature of cell signaling (200,201,311,312). In the developing eye, clusters of eight photoreceptor cells develop within a two-dimensional lattice that is seeded by the initial specification of the founder R8 cell from an epithelium of cells that initially are more or less equivalent. Before R8 specification, EGFR activity is required for cells to proliferate normally. EGFR activity serves in the mechanism that spaces R8 cells at regular intervals. EGFR activity (induced by a ligand made in R8 cells) is then required for the recruitment, G1 arrest, and differentiation of the other photoreceptor cells R1–7 (which differentiate into similar cell types but with distinct differences in both position and gene expression). Part way through this process (when five of the eight cells have been recruited into a cluster), EGFR activity is required to stimulate the G2/M-phase transition in a single round of mitosis of the undifferentiated cells not in a cluster. EGFR activity is also required to prevent apoptosis of these cells, so that a sufficient number of cells remain to form the mature ommatidium. Thus, the very same receptor is activated to achieve multiple different responses from an initially uniform group of cells. In particular, outer photoreceptor cells are induced to divide, survive, and differentiate by successive EGFR signals within a relatively short period of time (<1 d). These three responses inevitably must have different molecular signatures, representing three successive states of responsiveness. In this example, the molecular changes underlying the different responses are not known.

The control of appendage development in *Drosophila* by Hh signaling provides a clearer molecular example. In wing and leg imaginal discs (precursors to wings and legs), Hh is made in posterior cells and induces critical target genes, including the TGF- $\beta$  superfamily member Dpp, in nearby cells in a dorsoventral stripe running down the center of the disc (313,314). Target genes are not induced in posterior cells (in an autocrine manner) because these cells have previously been assigned a distinct identity through the heritable expression of the transcription factor Engrailed. Engrailed represses transcription of the Hh signaling pathway effector Ci. In anterior cells, Dpp is induced throughout the wing disc but only in dorsal regions of the leg discs, because Wg expression has already been activated in ventral cells (and is now induced by Hh signaling) and the Wg signaling pathway inhibits expression of Dpp (315–317). In other words, the different histories of exposure to earlier signals of posterior, dorsal, and ventral cells dictate whether they respond to an Hh signal at all and how they respond.

The idea of successive episodes of cell behavior fits easily with the acknowledged successive accumulation of mutations as cancer cells develop. The key expectation based on normal development is that the consequence of any one mutation will not always be the same. It will depend on what other mutations are present and on the environment of the precancer cell at the time. Some potentially important examples of this principle include cellular responses to activated Ras. The response of primary cells to activated Ras is to arrest cell cycling due to induction of p21 or p16, whereas cells that have already suffered a mutation in the Rb pathway (such as Rb itself) will not be inhibited by p21 or p16 and instead will be stimulated to proliferate through other actions of Ras (9,89). In colorectal cancer cells, activated Ras can further induce cyclin D1 expression, but Ras cannot do this if the Wnt/ $\beta$ -catenin pathway is blocked, presumably because the repressive effect of TCF factors is dominant (89). Thus, molecular responses to Ras depend on the status of other pathways. Although the status of other pathways can be determined by mutations, it could equally well be affected by normal signals from nearby cells or from maintained changes in gene expression, stemming from earlier, completed signaling events.

During cancer development, the effects of mutations are likely to be persistent, whereas most signals during normal development are likely to be terminated by changes in cell position, production of signals, or desensitization mechanisms that operate substantially on receptors for signals. The developing cancer cells may undergo signaling episodes that change their behavior in a stepwise fashion but they also carry through cumulative changes in signaling pathways, allowing for synergy among signaling pathways that might be less common in normal development and much less well studied in context. From numerous studies involving conditional expression of oncogenes in mice, it appears that the primary activation event inducing tumors must be maintained throughout the course of tumor development, or tumors will regress (309), possibly because the inducing mutation produces the same consequence (e.g., cell cycling) throughout development of the cancer or because it serves different roles as the cancer develops. The latter possibility is commensurate with the wide-ranging consequences of mutations affecting genes such as *Ras*, *Myc*, and *p53*.

### *Environmental Signals and Cellular Collaboration*

Normal cells build tissues that include balanced numbers of cells of different function. Although a large part of this collaboration is to ensure appropriate cell fates, signals are passed among the developing collaborators that ensure survival and proliferation of each cell. Sometimes signals prevent cell growth and instruct differentiation, or migratory signals, or instruct apoptosis to mold tissue architecture. The environment will impose different challenges to the development of cancer cells. In some cases, signaled cell movements will draw cells from a region containing proliferative signals into one containing inhibitory signals, differentiation signals, apoptotic signals, or cell death by other means. This might, for example, be characteristic of intestinal epithelium cells. In this situation, it might be most crucial first to block cell movements. In other situations, cell proliferation might be constrained largely by signals that limit cell cycling, perhaps by inducing terminal differentiation. Here, it would be essential for mutations first to prevent cell-cycle arrest if a cancer is to develop. Therefore, one might expect the types of mutations that initiate a path to cancer to differ among target cells according to their local environment.

At a later stage of cancer development, the influence of the cancer cells' original environment will be replaced with that produced by the cancer cells themselves. Factors produced by cancer cells are crucially important. This fact was appreciated a long time ago in terms of autocrine stimulation by production of growth factors by cancer cells and more recently by finding TSGs that appear to act in stromal cells around colorectal epithelial cell adenomas and cancers (318). This concept also extends to replacing factors that are present in a normal environment, especially survival factors. A recent illustration comes from the analysis of Rb mutant mice in which it was found that extensive apoptosis of Rb mutant cells can be rescued by creating mosaic mice that also include cells with functional Rb (311). The implication is that normal cells produce survival factors that Rb mutant cells cannot. It is possible that successful cancer development depends on maintaining a somewhat heterogeneous mixture of cells, including normal cells and different precancer cell derivatives that have different capacities to produce a changing mixture of growth and survival factors that may be required extracellularly at various stages of cancer cell development. This situation would be analogous to the cooperative interactions among cells that direct and sustain normal development. It would likely include mechanisms built into normal development, whereby cells recruit their own blood supply through angiogenesis. Thus, various traits of developing cancer cells might not depend on specific mutations but rather on intrinsic self-organizing attributes of groups of cells that are normally further regulated to mold development of tissues of precise size and cellular composition.

#### *Target Cells for Cancer Initiation and Progression: Stem Cells?*

It is extremely difficult to define the precise target cell where a cancer was initiated even when using specific promoters expressing oncogenes in mouse models. It certainly seems easier (and a more likely process) to convert a cell with strong proliferative potential to form a cancer than it is to convert a terminally differentiated cell; however, is it significantly easier to convert a stem cell into a cancer cell than another type of proliferating, undifferentiated cell? The answer to this question is not known. On one hand, some stem cells have properties of telomere maintenance and potentially limitless replication in a stable environment. On the other hand, the proportion of proliferating cells that are stem cells is generally very low, making stem cells a statistically less likely target to acquire relevant mutations.

While it is possible that stem cells are targets for colon cancers and BCC, induced by activation of Wnt and Hh pathways, respectively (282), colorectal tumors show that dysplastic cells and cells containing APC mutations are generally excluded from the base of crypts, where normal stem cells are thought to reside (319). One interpretation of these observations is that stem cells were not the target cells for this cancer. Other explanations are possible because not all stem cells may reside or need to be maintained in the base of crypts and because single mutationally altered (stem) cells could easily be missed in these analyses. In this situation and others, it is worth considering the possibility that the initial mutations do occur in stem cells and that a crucial consequence of the mutation is that stem cells can now survive in a different position. In other words, a mutation substitutes for a critical extracellular signal that is found in the microenvironment of the normal stem cell, normally confining it to that position (or niche). This is what appears to happen when the Hh signaling pathway is mutationally activated in somatic stem cells of the *Drosophila* ovary (320).

Hematopoietic stem cells are the best-characterized vertebrate stem cells, and several insights into stem cell contributions to cancer come from studying leukemias. It appears that leukemias can be induced by targeting mutational events, such as activation of Bcl-2 to restricted progenitor cells (non-stem cells) (321,322). This process is not very efficient, suggesting that, among many possible explanations, these cells have to adopt stem cell-like characteristics through additional specific mutations (321). Other studies imply that stem cells can be targets in human cancers. A specific translocation causing acute myeloid leukemia (AML) in a human was found in some hematopoietic stem cells, implying that is where they arose (321,323). Interestingly, the stem cells themselves were not leukemic, suggesting that further mutations occurred in their progeny.

These observations raise the interesting concept of cancer stem cells (321). The basic idea is that a stem cell would undergo at least one critical mutation, whereby many progeny are produced that can undergo additional critical mutations, with a number of ramifications. First, if the initial target stem cell is a long-term stem cell, i.e., one that divides over a long period of time, then there is an increased chance of accumulating environmentally induced mutations, as well as those induced during DNA replication. Second, a hierarchy may exist where short-term stem cells are derived from the long-term stem cell before giving rise to transit-amplifying (nonrenewing cells). This hierarchy could allow for the accumulation of different sets of mutations over a prolonged period of time in different derivatives of the original stem cell. These different cells could contribute to building a suitable environment for the eventual emergence of a single clone of a fully developed cancer cell. For example, hypothetically, the initial stem cell may be protected from apoptosis. One set of derivatives may initially aberrantly produce growth factors so that other derivative lineages, which have undergone a mutation affecting genomic stability, can initially proliferate. Later, a cell in this lineage may acquire a mutation that activates growth factor-signaling pathways autonomously and relieves its dependence on the cell lineage that aberrantly produces growth factor.

Some evidence suggests the existence of cancer stem cells, but whether they are common or rare is not known because this is generally hard to determine. In most cancers only a small proportion of cells (of the order of 0.1%) can form single-cell-derived colonies in soft agar or produce clones with other properties of cancer cells (321,324). This fact could be because only a specialized subset of cells can regenerate a tumor or because cells are homogeneous but have a low statistical chance of each of them doing this. For human AML these explanations were tested further by purifying AML cells into different pools according to cell-surface markers. Only those cells expressing markers characteristic of stem cells (<1%) were capable of seeding leukemias into mice (321,325). It is possible but certainly not proven that both the initiation and maintenance of some cancers may depend critically on a minor population of stem cells. These stem cells would not necessarily harbor all of the mutations found in some of their derivatives, but the cellular and mutational dynamics would make it likely that additional mutations would consistently recur to regenerate cells with a more complete cancerous phenotype. Eventually, of course, cells may sustain sufficient mutations such that the cancer no longer depends on the stem cells of origin and most cells become capable of seeding further cancerous growths.

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# 10

## Estrogen Action and Breast Cancer

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Jennifer E. Fox, Hong Liu, and V. Craig Jordan

### Introduction

Breast cancer is the most common cancer affecting women worldwide (1). In the United States alone, an estimated 211,000 new cases of breast cancer will be diagnosed in 2003 (2). It is estimated that 1 in every 8 American women will be diagnosed with breast cancer within the course of her lifetime (2). With more sensitive and accurate means of early detection and an ever-increasing number of drugs available to treat breast cancer, it is likely that women diagnosed today will live longer and may need more than one type of cancer therapy. Many cellular factors mediate breast transformation and tumor growth including growth factors, members of phosphorylation signaling cascades, oncogenes, and nuclear hormone receptors. While each of these factors has a role in the development of breast cancer, the steroid hormone estrogen is the primary promotional factor. Epidemiologic evidence has shown that a woman's overall lifetime exposure to endogenous estrogen, increased by early menarche, late menopause, and nulliparity, is the primary risk factor for developing breast cancer (3).

In 1896, George Beatson demonstrated that removal of the ovaries from a premenopausal woman with breast cancer could lead to a dramatic improvement in the course of the disease (4). However by 1900, Stanley Boyd (5) had demonstrated, in perhaps the first clinical trial, that only 1 in 3 premenopausal women could anticipate disease control after ovariectomy. The reason for this conundrum, now known to be the selective hormonal sensitivity of breast cancer, would not be discovered until 60 yr later, when Jensen and Jacobson (6) described the target-site specificity of estradiol in the immature rat. Their classic experiment showed that after an injection of [<sup>3</sup>H]estradiol, the radioactive steroid was bound to, and retained by, known estrogen target tissues, such as uterus, vagina, and pituitary gland. By contrast, estradiol was not retained by nontarget tissues, such as skeletal muscle. These observations led Jensen to postulate that an estrogen receptor (ER) present in estrogen target tissues must sequester the steroid specifically and initiate the cascade of biochemical events associated with estrogen action in that tissue. Increased estrogen exposure is the most important risk factor for the initiation and progression of breast cancer. Therefore, the ERs ER- $\alpha$  and ER- $\beta$ , which mediate estrogen action, have been well studied as both predictors of hormone sensitivity in breast cancer and crucial targets for anticancer drugs.

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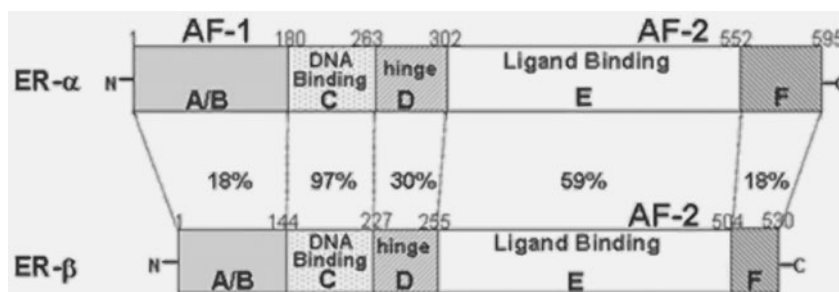


Fig. 1. Schematic comparison of human ER- $\alpha$  and ER- $\beta$  protein structure. The structural domains (A/B, C, D, E, and F), the LBD, the DBD, and transactivation domains (AF-1 and AF-2) are shown. The percentage of amino acid identity shared is indicated for each domain (28,301).

## Biology of Estrogen Receptors

As the prime mediator of estrogen action in the body, ERs bind estrogen and activate transcription of estrogen-responsive genes in target tissues, resulting in growth and differentiation of cells. In the mid-1960s, ER- $\alpha$  was the first ER shown to be soluble and not membrane bound (7,8). However, the cloning of the ER- $\alpha$  gene (9,10), along with ensuing advances in molecular biology and genetics in the 1980s, led to a wealth of new knowledge concerning the structural biology and pharmacology responsible for ER- $\alpha$ -mediated gene transcription. The discovery, in 1996, of a second distinct ER (ER- $\beta$ ), expressed in a different profile of tissues, has led to more detailed studies of the role each of these receptors plays in breast cancer (11–13). Although the physiologic significance of the existence of two or more ERs is not clear, differences in activity and tissue/cell distribution (14,15) may explain the wide-ranging activity of estrogens and the tissue-specific effects of selective ER modulators (SERMs) and estrogen-modulating drugs.

### Structure of ER- $\alpha$ and ER- $\beta$

ER- $\alpha$  and ER- $\beta$  are both members of the nuclear receptor superfamily, which includes steroid hormone receptors [glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor (PR), androgen receptor, and ERs], thyroid and retinoid hormone receptors, vitamin D receptor, and a large number of orphan receptors for which no ligands have been identified (16–18). Each receptor is named for its specific ligand, with the exception being orphan receptors, which have been placed in the nuclear receptor superfamily based on sequence homology but have no known endogenous ligand (18). Nuclear receptors share a conserved structure with the most homology found in the DNA-binding domain (DBD) and the least homology in the ligand-binding domain (LBD) regions (Fig. 1) (19,20). Each of these receptors functions as ligand-inducible transcription factors that initiate mRNA transcription by binding to DNA response elements located in the promoter region of responsive genes. Recent studies tracing the evolutionary origins of receptors have shown that the ER was the first ancestral member of the nuclear receptor superfamily (21). The cloning of ER- $\alpha$  was followed by the cloning of ER- $\beta$  first from rat prostate (11), and subse-

quently human (22) and mouse (23). ER- $\beta$  binds to DNA in a manner similar to ER- $\alpha$  (23–26), associates with coactivators (23,24), and activates estrogen response element (ERE)–dependent gene expression in a hormone-dependent manner (11,22–24,26). In addition, ER- $\beta$  can form a heterodimer with ER- $\alpha$  on DNA that enhances ERE promoter activity (24). The human ER- $\alpha$  protein is composed of 595 amino acids with a molecular weight of approx 67 kDa (17,27). The most abundant isoform of ER- $\beta$  is a 54 kDa protein (25), which binds estradiol with an affinity (0.5 nM) similar to ER- $\alpha$  (0.2 nM) (15,17). The two ERs are located on different chromosomes and share some similarities in function and protein structure including a highly conserved DBD (97%) and LBD (59%) (14,22,28). ER- $\alpha$  and ER- $\beta$  are composed of multiple interactive functional domains, including the N-terminal A/B domain, referred to as activation function-1 (AF-1); the C domain, referred to as the DBD; and the C-terminal E domain, referred to as the LBD, which also contains AF-2 (Fig. 1) (29,30).

### AF-1 Domain

The N-terminal A/B domain of ER- $\alpha$  (the AF-1 domain) is composed of 180 amino acids compared with 144 amino acids for ER- $\beta$  (28). AF-1 is a ligand-independent region primarily involved in protein–protein interactions and transcriptional activation of target genes (31,32). ER- $\alpha$  and ER- $\beta$  proteins differ most markedly in the AF-1 domain, in which they share an 18% homology. The divergence in structure and lack of homology in the A/B domains may explain the observation that ER- $\alpha$  and ER- $\beta$  are able to activate the transcription of different sets of specific estrogen-responsive genes (33–37). Experiments comparing the transcriptional activation of ER- $\alpha$  AF-1 vs ER- $\beta$  AF-1 have shown that the AF-1 of ER- $\alpha$  is much more active at stimulating a wide variety of ERE-containing genes in different cell lines (38). Conversely, the AF-1 of ER- $\beta$  has been shown to have negligible ability to activate ERE-reporter genes (38). The AF-1 of ER- $\alpha$  contains two distinct regions that are necessary for 17 $\beta$ -estradiol (estradiol) or tamoxifen-stimulated activation of ERE-reporter genes, whereas the AF-1 of ER- $\beta$  contains neither of these two interacting regions and responds to tamoxifen only as an antagonist (35,39). This may explain the disparity with which ER- $\alpha$  and ER- $\beta$  AF-1 domains activate transcription of ERE-containing genes in response to various ligands. Different portions of AF-1 in ER- $\alpha$  are required for tamoxifen and estrogen agonist activity (40). Deletion mutagenesis studies of ER- $\alpha$  AF-1 have shown that the first 40 amino acids are not required for transcriptional activity of ER- $\alpha$  (41). Further analysis showed that amino acids 41–64 are crucial for tamoxifen-stimulated ER- $\alpha$  activity but not for estradiol-stimulated activity (41).

AF-1 can act independently, but, as shown in MDA-MB231 human breast cancer cells and HEC-1 human endometrial cells, AF-1 must synergize with AF-2 for full ER-mediated activity (41–43). Many factors enhance AF-1 potentiation of AF-2 activity, including the presence of agonistic ligands such as estradiol or tamoxifen, which stimulate direct or indirect AF-1/AF-2 interactions. Coactivator proteins such as p68/p72 interact with the AF-1 domain and enhance the ability of tamoxifen and estradiol to stimulate ER transcriptional activity (44). In addition, coregulators that associate with ligand-bound ER, such as steroid receptor coactivator-1 (SRC-1), also enhance AF-1/AF-2 interaction (45). ER- $\alpha$  and ER- $\beta$  have opposite effects in regulating nonclassic (not through interactions with ERE) gene activation, such as in promoters containing activating protein 1 (AP-1) elements (37). AP-1 elements do not directly bind ER but, rather, bind members of the Jun/Fos family of transcription factors (46). Nevertheless,

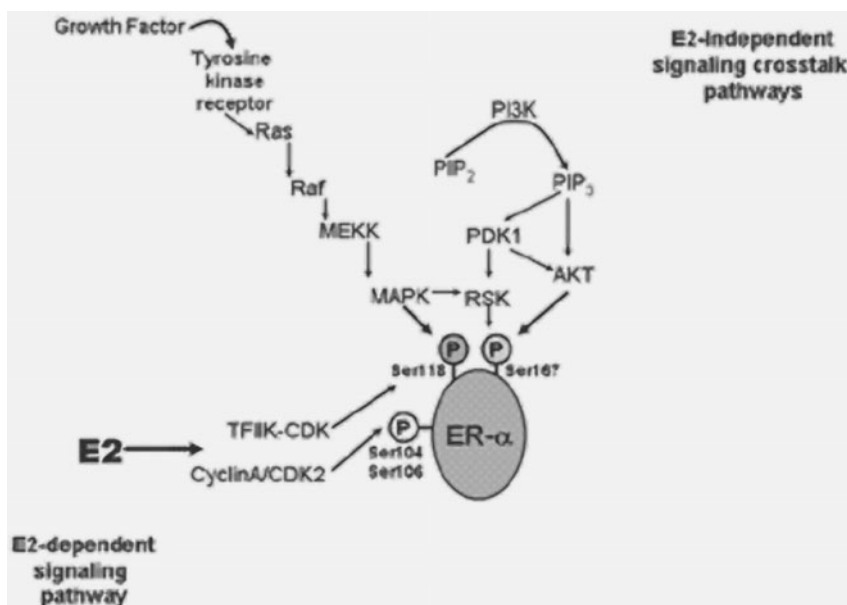


Fig. 2. Phosphorylation of key ER- $\alpha$  Ser residues by estradiol-induced signaling pathways and estradiol-independent pathways.

ER increases the intrinsic transcriptional activity of Jun/Fos at AP-1 sites, and ER agonists are able to activate AP-1 target genes (47). ER- $\alpha$  activation of AP-1 can occur through interactions between the AF-1 and AF-2 domains or through the AF-2 alone, while ER- $\beta$  activates AP-1 sites in an AF-1/AF-2-independent manner requiring only a DBD (37). The AF-1 domain of ER- $\alpha$  recruits SRC/p160 coactivators, which then interact with the CBP/p300 coactivators recruited by Jun/Fos, when bound to AP-1 sites (48). Therefore, ER- $\alpha$  AF-1 and Jun/Fos form a “tethered” complex together with other coactivators that results in enhanced transcriptional activity of the AP-1 signal-transduction pathway (48,49).

The AF-1 domain of ER is potentiated by the phosphorylation of various Ser residues (Fig. 2). Phosphorylation of these key Ser residues is induced by both estrogen-dependent and estrogen-independent pathways (Fig. 2). For example, phosphorylation of Ser 104, 106, and 118 is enhanced after estradiol binding to ER- $\alpha$  (50). The most dramatic increase in phosphorylation, occurring at Ser118, has been shown to potentiate AF-1 function (51,52). Estradiol binding also results in cyclin-A and cyclin-dependent kinase 2 (Cdk2) phosphorylation of Ser104 and Ser106 but not Ser118 (53). In the absence of estradiol, both ERs show an increase in transcriptional activity stimulated by mitogen-activated protein kinase (MAPK) phosphorylation of key residues in the AF-1 region (54,55). Researchers have shown that estradiol enhances the activity of c-src, which then activates the MAPK pathway leading to phosphorylation of Ser118 (56–58). Alternatively, in an estrogen-independent manner, epidermal growth factor receptor (EGFR) and other types of tyrosine kinase signaling activate the MAPK phosphorylation cascade, which then phosphorylates Ser118 (54,59–61). Therefore, Ser118 can be phosphorylated by both estrogen-dependent and -independent pathways (62,63) (Fig. 2).

Ser167 is phosphorylated in an estrogen-independent manner by p90 ribosomal S6 kinase (Rsk), a member of the MAPK signaling pathway (60,62,63). Ser167 can be simultaneously phosphorylated by AKT, owing to cross talk between signaling pathways in which PDK1 stimulates the activity of both Rsk and AKT (59,61,64–66). Similarly, MAPK phosphorylates Ser106 and Ser124 of ER- $\beta$ , resulting in estrogen-independent recruitment of coactivators and increased transcriptional activity (28,67).

### *DNA-Binding Domain*

The C domain, ER- $\alpha$  amino acids 181–263 and ER- $\beta$  amino acids 145–227, functions as the DBD, which is the most highly conserved region of nuclear hormone receptor superfamily members (68,69). Of the nine conserved cysteine residues of the DBD, eight comprise two Zn<sup>2+</sup> finger structures that are crucial for receptor dimerization and specific DNA binding (70–72). The DBD of ER- $\alpha$  and ER- $\beta$  shares 97% sequence homology, with identical sequences comprising a region called the P-box. The P-box is a region of six amino acids (CEGCKA) at the C-terminal base of the first zinc finger that is critical for site-specific DNA sequence recognition (73). The specific DNA sequence located in the promoter region of responsive genes, which ER- $\alpha$  and ER- $\beta$  bind, is called the ERE. The ERE is a perfect palindromic repeat with a consensus sequence of (5'-GGTCANNNTGACC-3'), which is specifically recognized by the ER DBD. The P-box is partially responsible for recognizing the exact ERE sequence. In fact, deletion mutagenesis studies have shown that an ER mutant with only three amino acid substitutions in the P-box can no longer bind an ERE but, instead, specifically recognizes and binds the glucocorticoid response element (74). While the exact amino acid sequence of a hormone receptor's P-box influences DNA binding, another region of the DBD, the D-box, also contributes to site-specific binding. Hormone-response elements, such as ERE, are palindromic repeats, which reflects the fact that the dimerization of receptors is an important component of DNA binding.

The D-box is the receptor dimerization interface located at the N-terminus of the second zinc finger of the DBD (68,69). ER- $\alpha$  and ER- $\beta$  can either homodimerize or heterodimerize. Dimerization leads to D-box interactions that stabilize DBD-ERE binding and can enhance binding to imperfect EREs, which expands the number of sequences with which the ER can interact (75). The three-dimensional (3D) crystal structure of the ER- $\alpha$  DBD bound to an ERE (72,76,77) shows that two molecules of the DBD sit in the adjacent major grooves from one side of the DNA double helix. The side chains of Glu 203, Lys 206, Lys 210, and Arg 33 interact with the central six bp of AGGTCA by hydrogen bonds. Tyr 195, His 196, Tyr 197, Arg 211, Arg 234, Lys 235, Glu 238, and Arg 241 contact the phosphate backbone of ERE. The crystal structure data further support the results from biochemical and mutational studies. In addition, there is weak dimerization activity within the minimal region for DNA binding (78,79), which is also observed in the DBD crystal structure.

### *Hinge Region*

The D domain, ER- $\alpha$  amino acids 264–302 and ER- $\beta$  amino acids 228–255, is a hinge region that interacts with a chaperone protein, heat-shock protein 90 (hsp90) (30). Hsp90 associates with ER, rendering the unliganded ER inactive and stable, which is crucial for the high hormone-binding affinity of ER. The hinge region has been shown to associate with a coactivator L7/SPA in the presence of the antiestrogen tamoxifen

(80), which may enhance the partial agonist activity of tamoxifen. In addition, the coactivator PGC-1 interacts in a ligand-independent manner with the hinge region of ER and interacts in an agonist-dependent manner with AF-2, enhancing the transcriptional activity of ER and mediating cross talk with peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) (81).

### *Ligand-Binding Domain*

The COOH-terminal E domain, also known as the LBD, ER- $\alpha$  amino acids 303–552 and ER- $\beta$  amino acids 256–504, is the largest domain of the ERs. The F-domain is not conserved in the nuclear receptor superfamily, and ER- $\alpha$  and ER- $\beta$  share only 18% homology in this domain. The F domain has been implicated in differential regulation of estrogen- and antiestrogen-responsive transcription in a cell-type-dependent manner (82). ER- $\alpha$  and ER- $\beta$  share 59% genetic homology in domain E, which contains regions responsible for specific ligand binding, coactivator and corepressor recruitment, and hormone-dependent transactivation activity (AF-2 domain) (16,83–85). The first 3D crystal structure of a steroid hormone LBD was for ER- $\alpha$  (86). Crystal structures of the ER- $\alpha$  LBD bound to agonists such as estradiol and diethylstilbestrol (DES) and to SERMs such as raloxifene and 4-OH tamoxifen have been determined (86–88), giving a clear view of how agonists and antagonists induce different conformational changes in the LBD.

The ER- $\alpha$  LBD has 12  $\alpha$ -helices (H1–H12) that, on agonists such as estradiol and DES binding, fold into a three-layered  $\alpha$ -helical sandwich and two-stranded antiparallel  $\beta$ -sheets (S1 and S2). The central core of three helices is surrounded by two layers of helices creating a hydrophobic ligand-binding cavity, which is larger than that of most other nuclear hormone receptors (28,89,90). The comparative expansiveness of the ER- $\alpha$  ligand-binding cavity explains why estradiol does not occupy the entire cavity and why ER is able to bind a wide variety of steroids and environmental estrogens. When agonists such as estradiol and DES bind ER- $\alpha$ , helix 12 (H12) is positioned over the ligand and across the ligand-binding cavity in a groove created by H3, H5/H6, and H11. This configuration of the agonist-bound ER- $\alpha$  LBD exposes the coactivator-binding region of ER- $\alpha$  to the LXXLL recognition motifs found on coactivators such as the p160/SRC class of coactivators (12). Antagonists and SERM, such as raloxifene and 4-OH tamoxifen, also bind ER- $\alpha$ , but induce very different conformations of the LBD in which H12 is placed in a position to block ER's coactivator interaction site (86,87). As a result, H12 positions itself between H5 and H3, masking amino acids in the LBD that are critical for ER- $\alpha$  interaction with SRC coactivators (91). Both raloxifene and 4-OH tamoxifen have alkylaminoethoxy phenyl side chains, which extend out of the ligand-binding cavity and interact with Asp 351 in H3 of ER- $\alpha$ , indicating that this residue may be critical for antagonist activity. In fact, a mutation of Asp351 to Tyr has been discovered in breast cancer cells (92) that have become resistant to the antagonistic effects of tamoxifen and raloxifene (93,94). The differential displacement of H12 to cover an agonist ligand and allow the ER- $\alpha$  recognition site to be available for binding coactivators or for an antagonist ligand to displace H12 and obscure the coactivator recruitment site appears to be the key component of ER- $\alpha$ 's discrimination between agonist and antagonist ligands.

Further clues as to how ER- $\alpha$  and ER- $\beta$  are able to discern agonists from antagonists have been found by analyzing the X-ray crystallography of ER- $\beta$  LBD bound to the isoflavonoid genistein and the SERMs raloxifene (95). The overall 3D structure of the

ER- $\beta$  LBD bound to the antagonist raloxifene is similar to that of ER- $\alpha$ , with H12 displaced to block coactivator binding to the ER- $\beta$  LBD (28,85,95). Conversely, when genistein binds the ER- $\beta$  LBD, H12 does not adopt an agonist conformation, but, rather, H12 is displaced to the typical antagonist conformation in which it obscures coactivator recruitment to the genistein-ER- $\beta$  LBD (96).

Some of the most innovative recent efforts to dissect the actions of ER- $\alpha$  from ER- $\beta$ , particularly as it pertains to ligand responsiveness, have produced ER- $\alpha$  and ER- $\beta$  subtype-specific ligands. Such ligands include propyl pyrazole triol, an ER- $\alpha$ -selective agonist that has been shown to exert only ER- $\alpha$  selective agonist effects *in vivo* (97), and methyl-piperidino-pyrazole, an ER- $\alpha$ -selective antagonist (98). Another selective ligand is 5,11-*cis*-diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol (THC), which exerts opposite effects on the transcriptional activity of ER- $\alpha$  and ER- $\beta$  (99). THC is an ER- $\alpha$  agonist and an ER- $\beta$  antagonist. THC does not have a bulky substituent side chain, as used by raloxifene and 4-OH tamoxifen to displace H12 from an agonist conformation. Rather, it has been shown that THC exerts its effects through differential coactivator (SRC-1, SRC-2, and SRC-3) recruitment to THC-bound ER- $\alpha$  and ER- $\beta$  (99). Recently, the 3D crystal structure of the ER- $\alpha$  LBD bound to THC and a fragment of the coactivator SRC-2 (GRIP-1) and ER- $\beta$  LBD bound to THC has been determined (100). The 3D structure of ER- $\alpha$  and ER- $\beta$  bound to THC shows that THC stabilizes the agonist conformation of the ER- $\alpha$  LBD that permits coactivator recruitment, while THC stabilizes the antagonist conformation of the ER- $\beta$  LBD preventing coactivator recruitment. These ER-subtype-selective ligands are not only useful for examining the biologic functions of ER- $\alpha$  and ER- $\beta$ , but may also be a blueprint for drugs that selectively block responses mediated by only ER- $\alpha$  or ER- $\beta$ .

In addition to AF-1 and AF-2, a third activation domain (AF-2a), located between amino acids 282 and 351 of ER- $\alpha$ , has been identified within the boundary of the D and E domains (101,102). *In vitro* studies have shown that human TATA binding protein-associated factor (TAF<sub>II</sub>30) directly interacts with the ER- $\alpha$  AF-2a domain in a hormone-independent manner to enhance ER-mediated transcription (103). The enhanced transcription, owing to binding of TAF<sub>II</sub>30 to AF-2a, may be the mechanism for the autonomous transactivation activity of AF-2a in yeast and mammalian cell systems (101,102).

Since each domain of ER plays a specific role, it is not surprising that individual amino acids within these domains contribute extensively to the activity of the receptor. Most knowledge on the role of particular amino acids from ER has been gained from extensive mutational analysis of ER mutants found in human tumors and from site-directed mutagenesis experiments (Fig. 3). Mutational analysis has shown that each step in the transcriptional activation of ER-dependent genes (ligand binding, ER dimerization, DNA binding, cofactor recruitment) is influenced by the phosphorylation status of ER. For example, it has been shown that Ser 104, 106, 118, and 167 are critical phosphorylation sites, and mutations in these sites lead to an inactive ER (50,104). Mutation of the Y537 phosphorylation site results in a conformational shift of H12 leading to an ER that binds coactivators in the absence of ligand and, as a result, is constitutively active (105). Conversely, it has been reported that the Y537X mutation increases NCoR binding in the presence of all SERMs and blocks AF-1 activity; the effects of this mutation are reversed when coupled with the L379R mutation (106). When mutated, residues located in the DBD—E203A, G204A, and K206A—abolish ER- $\alpha$  binding to ERE sites of responsive genes, resulting in an ER that can only acti-

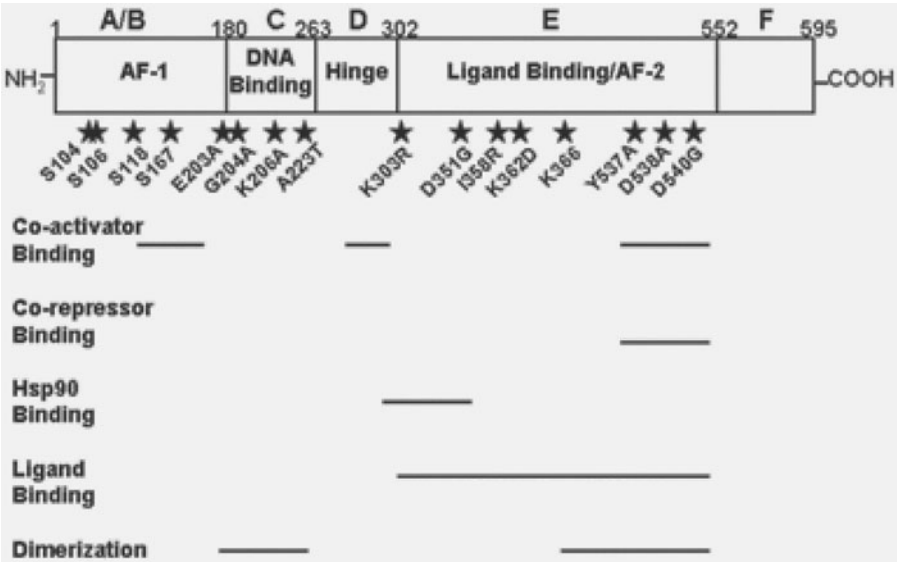


Fig. 3. Schematic diagram of human ER- $\alpha$  protein structure with mutations of key amino acids indicated by stars. Horizontal bars highlight areas of binding or interaction with ligands and coregulators.

vate genes through the “tethered” nonclassic AP-1 pathway (74). Likewise, the mutation pair E207A/G208A abolishes classic ER signaling through ERE binding, leaving ER to signal only through AP-1 elements (107,108). An ER mutation identified in premalignant breast lesions, K303R, confers a higher binding affinity for estradiol and increased binding of SRC-2 resulting in estrogen sensitivity (109). Residue D351, originally found to be mutated (D351Y) in breast tumor cells that had become tamoxifen stimulated over time (92,110), has been found to be important in mediating the partial agonist activities of tamoxifen. The D351G mutation allosterically silences AF-1 activity in the TAM-ER complex, resulting in the loss of partial agonist activity of TAM and maintenance of TAM antagonist activity (94,111). The natural mutation D351Y shows weak binding to coactivators such as GRIP1 and SRC-1 and reduced binding to corepressor proteins, which leads to enhanced TAM agonist activity and changes Ral-like compounds from antagonists to partial agonists (93,112,113). Mutation of K366A blocks ER binding to the SRC-1 coactivator while allowing ER to bind the RIP140 coactivator (114). The L379R mutation decreases NCoR binding and increases AF-1 activity in the presence of ICI and raloxifene (106). The L384M mutation in the LBD of ER- $\alpha$  results in a ligand-binding shift wherein the mutant L384M/ER- $\alpha$  now binds ligands that were specifically recognized only by ER- $\beta$  (115). The triple mutation L508E/L512E/L515E inhibits ER dimerization, resulting in loss of ligand-dependent transcriptional activity and transrepression (116). Mutations within the LBD such as D538A have been shown to negate the partial agonist activity of TAM and facilitate degradation of the TAM-D538A/ER- $\alpha$  complex (117). The D540G mutation inverts the activity of some SERMs, allowing ER to be activated by TAM and ICI 164,384 but not by estrogens (118).



Studies have shown that point mutations within the DBD altered ER activation of ERE and AP-1 sites in response to estradiol, tamoxifen, and ICI 182,780 (119). Site-directed mutagenesis studies have provided information about the importance of the AF-1 of ER- $\alpha$  for ER-mediated gene activation. For example, when ER- $\alpha$  is mutated to delete the entire AF-2 core, leaving only AF-1 activity, the resulting mutant ER- $\alpha$  perturbs normal estradiol signaling by becoming constitutively associated with corepressors such as nuclear receptor corepressor (NCoR)/silencing mediator of retinoid and thyroid receptor (SMRT) and histone deacetylases (HDAC) (120). ER mutants such as these have been found expressed in human tumors (121,122).

### **Ligand-Dependent Activation of ER- $\alpha$**

ER- $\alpha$  and ER- $\beta$  activate target gene expression by classic, ERE-mediated, and nonclassic pathways. EREs were first discovered in 1986 in the 5'-flanking region of the *Xenopus* vitellogenin A2 gene (123). Therefore, it is not surprising that the classic ERE-mediated pathway is the best-characterized mechanism for ER activation of responsive genes. The natural ligand for ER- $\alpha$  and ER- $\beta$  is 17 $\beta$ -estradiol (estradiol). Estradiol is synthesized from cholesterol and is a hydrophobic molecule capable of entering the cell and nucleus by diffusion. In the absence of ligand, ER is sequestered in target cell nuclei bound to an inhibitory chaperone/hsp complex including hsp70 and hsp90 (95,124). In the classic model of ER activation, estradiol binding induces conformational changes in the ER tertiary structure, leading to (1) dissociation of the chaperone/hsp protein complex, (2) dimerization of ligand-bound ER within the regulatory regions of target genes, and (3) helix 12 realignment over the agonist-bound LBD to form a hydrophobic cleft with exposed residues that serve as docking sites for coactivator proteins. Coactivator proteins interact with ligand-bound ER and enhance ER-activated gene transcription through chromatin remodeling, histone acetyltransferase (HAT) activity, and contact with general transcriptional machinery. Ligands that bind ER are able to modulate estrogen-responsive genes, and one mechanism by which ligands exert differential effects on ER activity is through cofactor association. When comparing ligands capable of binding ER to estradiol, such as the plant estrogen genistein, the synthetic pharmaceutical DES, and the estrogenic organochlorine compound bisphenol A it has been shown that the specific ligand bound can control the strength of ER-regulated gene activation simply by influencing the cast of coactivators recruited to ER (125). Therefore, activation of the ER not only relies on ligand binding, but is perhaps equally influenced by coactivator protein binding.

Initial evidence for the presence of coactivators came from experiments describing transcriptional interference or "squenching" when one overexpressed receptor inhibited the activity of other receptors without any direct interaction or competition for DNA binding (126,127). The investigators hypothesized that the receptor's transcriptional activity may be inhibited owing to a limiting cellular factor. ER-protein interaction screens have revealed an extensive group of proteins (Table 1) classified as coactivators based on shared characteristics. For example, these proteins have the ability to bind specifically to ER in an agonist/ligand-dependent manner; coactivator-ER binding is negatively affected by antiestrogens such as ICI 182,780, and binding of the coactivator enhances ER transcriptional activity (128,129). After binding estradiol, ER undergoes a conformational change aligning H12 in a specific agonist confirmation that exposes a docking site for various coactivator proteins (86). Recruitment and docking of ER-associated coactivators is mediated by a conserved

**Table 1**  
**Proteins Classified as Coactivators and Their Functions**

CoActivator	ER domain binds	Function	Reference
AIB3 (ASC-2)	NA	Gene found amplified in breast tumors; interacts with SRC-1 and CBP/p300 to enhance ligand-dependent transcription	(305,306)
ARA70	LBD	Increases transcription; interacts with p/CAF and TFIIB; HAT activity	(307)
BRG-1	LBD	Interacts with SWI/SNF ATP-dependent chromatin remodeling complex	(308)
CARM-1	NA	Enhances transcription via intrinsic methyltransferase activity	(309)
CBP	LBD	HAT activity, cointegrates signaling with SRC-1, SRC-2, SRC-3, and basal transcription machinery	(144,310)
E6-AP	NA	Enhances hormone-dependent transcription, ubiquitin-ligase activity	(311)
ERAP 140	LBD	HAT activity; interacts with p300, TFIIB, and TBP; enhances interaction of AF-1 and AF-2 domains	(230)
L7/SPA	LBD and hinge region	Increases TAM agonist activity with no effect on E <sub>2</sub> or pure antiestrogen activity	(80)
MNAR	NA	Modulates nongenomic activity of ER by activating Src/Erk phosphorylation cascades	(156)
NSD1	LBD	Mixed coactivator/corepressor; contains separated activation and repression domains that interact differentially with liganded and unliganded receptors; chromatin-modifying ATPase	(312)
p68/p72	AF-1	Interacts with CBP and SRA; enhances TAM and E <sub>2</sub> -induced transcriptional activity	(44)
p300	NA	Binds SRC-1; HAT activity	(144,313)
p/CAF	NA	Binds CBP/p300; HAT activity	(132,137,314)
PELP-1	AF-2	Enhances estrogen-dependent transcriptional activation from EREs; interacts with p300 and CBP	(315)

PGC-1	Hinge region and AF-2	Interacts in a ligand-independent manner with hinge region of ER; interacts in an agonist-dependent manner with AF-2; mediates signaling cross talk between ER- $\alpha$ and PPAR- $\gamma$	(81)
PRMT-1	NA	Histone methyltransferase activity; methylates Arg3 of helix 4 and facilitates acetylation of helix 4 by p300	(139,316)
RIP140	AF-2	Enhances transcriptional activity in a promoter-specific manner	(317,318)
SRA	NA	Assembles a ribonucleoprotein coactivator complex by mediating contacts between AF-1 and the SRC/p160-AF-2 complex	(146,147,319)
SRC-1 (ERAP160, nCoA1, p160)	LBD	Enhances transcription by interacting with AF-1 and AF-2; interacts with p300, TFIIB, and TBP; coactivates activity of AP-1 and NF- $\kappa$ B; HAT activity; overexpression rivals SMRT repressor activity	(31,114,132)
SRC-2 (TIF2, GRIP-1)	Helix 12/LBD	Enhances transcription by strong endogenous activational activity; interacts with CBP	(320)
SRC-3 (AIB-1, ACTR, RAC3, TRAM-1, pCIP)	LBD	Interacts with p/CAF and CBP/p300; enhances transcriptional activity of ER- $\alpha$ > ER- $\beta$ ; HAT activity	(132,133)
TIF1 (TIF-1 $\alpha$ )	AF-2	Enhances transcription in ligand-dependent manner by modifying chromatin structure	(148,321)
Tip60	LBD	Enhances transcriptional activity	(322)
SWI/SNF1 (SPT6)	LBD	Enhances transcriptional activity of ER in vitro by remodeling chromatin	(323,324)
SWI2/SNF2	NA	Enhances transcription through remodeling chromatin; interacts with SRC-1	(325)
TRAP220 (TRIP2, PBP)	LBD	Enhances transcriptional activity; thought to interact with coactivators and general transcriptional machinery; gene found overexpressed in human breast tumors	(326)
Zac1	AF-2	Mixed coactivator/corepressor with differential regulation of nuclear receptors in different cell lines; enhances transcription in a hormone-independent manner by binding SRC-2 and CBP; involved in regulation of apoptosis and cell-cycle control	(327)

NA, not available

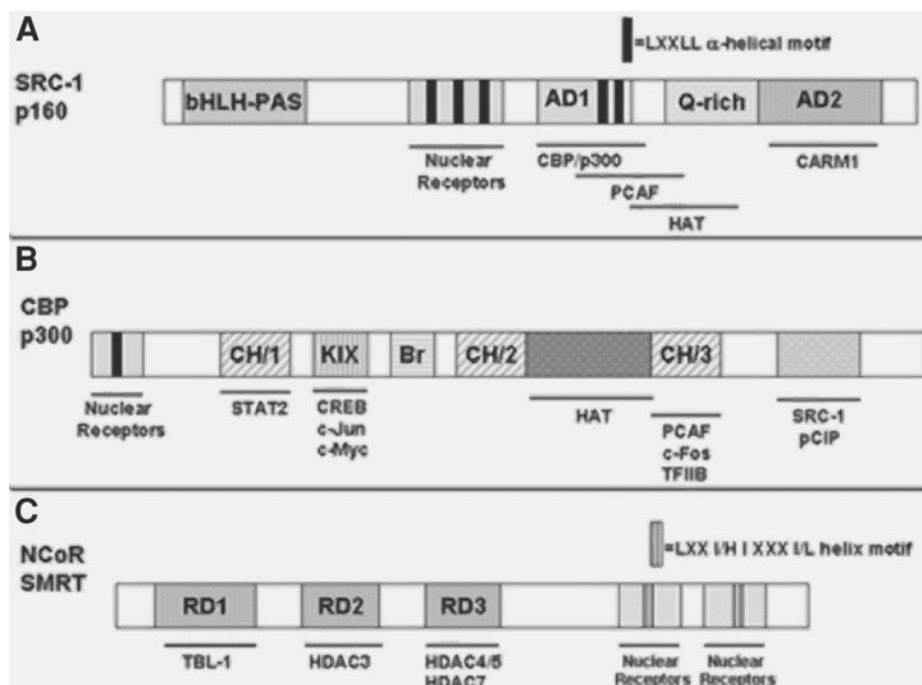


Fig. 4. Structure of coactivators (SRC-1/p160 and CBP/p300) and corepressors (NCoR, SMRT). (A) The basic structure of SRC-1/p160 contains a basic helix-loop-helix (bHLH) motif and a Per-Arnt-Sim (PAS) homology region. The nuclear receptor interaction domain contains three LXXLL motifs. Areas of interaction with other cofactors as well as HAT activity are indicated below the structure (143,302,303). (B) The basic structure of CBP/p300 also contains a nuclear receptor interaction domain with an LXXLL recognition motif. The structure also has three zinc-finger regions (CH/1, CH/2, CH/3), a bromodomain (Br), and a KIX domain that interacts with CREB (30,87). (C) The basic structure of the corepressors NCoR and SMRT contains three repressor domains (RD1, RD2, RD3) and two nuclear receptor interaction domains with the extended helical motif LXX I/H I XXX I/L. RD1 interacts with TBL-1, which then recruits HDAC1 and HDAC2. Other HDACs interact with regions of RD2 and RD3 and are indicated below the structure (168,302,304).

amphipathic helix with an LXXLL (L is leucine and X is any amino acid) motif, called the nuclear recognition motif box (NR box) (128). Among the first coactivators identified were the SRC family of coactivator proteins (31,114,130–134).

The SRC family consists of 3 homologous proteins, SRC-1, SRC-2, and SRC-3, each expressing 3 conserved LXXLL/NR box motifs for binding the AF-2 domain of ER- $\alpha$  and other nuclear receptors (Fig. 4). SRC-1 (p160, NCoA), SRC-2 (TIF-2, GRIP-1, NCoA2), and SRC-3 (AIB1, p/CIP, ACTR, RAC3, TRAM-1) each coactivate ER- $\alpha$ , in an agonist-dependent manner, through ER- $\alpha$ /AF-2 docking site and SRC/NR box interactions. Since SRC-1, SRC-2, and SRC-3 show similar affinities for ER- $\alpha$ , it has been hypothesized that the main determinant of which SRC proteins is complexed with ER is the relative abundance of specific coactivators within various target tissues (135,136). SRC coactivators enhance ER- $\alpha$  activity by mediating interactions between the AF-1

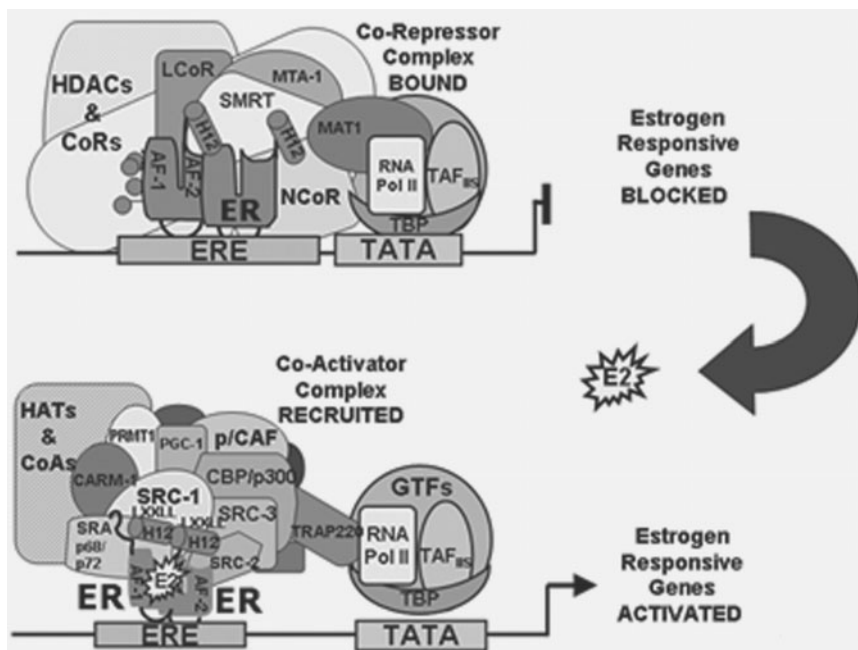


Fig. 5. Promoter activity at estrogen-responsive genes. In the absence of estradiol, a corepressor complex associates with ER and blocks transcription of ERE-containing genes. In the presence of estradiol, the corepressor complex dissociates from ER and coactivators are recruited to ER and interact with general transcription factors, which serve to enhance ER-mediated transcription.

and AF-2 domains of ER. SRC-1 and SRC-3 possess HAT activity useful for chromatin remodeling and ER-directed transcriptional initiation (132,137). In addition, SRC proteins can further influence chromatin remodeling by recruiting methyltransferase proteins (138,139). This interaction illustrates the most important function of SRC coactivators, which is to recruit a host of other transcriptional coactivators and HATs to the ER complex of estrogen-regulated target genes (Fig. 5) (140).

Among the vital coactivators recruited to ligand-bound ER by SRCs is CBP/p300 (cyclic adenosine monophosphate [cAMP] response element-binding protein, or CREB) and p/CAF (p300 and CBP-associated factor), which binds CBP/p300 indirectly to SRC (131,137,141). CBP preferentially binds SRC-3 over SRC-1 and SRC-2 and serves to enhance ER transactivation through HAT activity and as a molecular scaffold for more extensive coactivator recruitment (Fig. 5) (128,141–143). The N-terminus of CBP/p300 contains an NR box that allows it to interact directly with nuclear receptors (142), and the C-terminal is responsible for SRC binding (144,145). SRC's C-terminus serves to recruit other coactivators such as coactivator-associated arginine methyltransferase-1 (CARM-1) and protein arginine methyltransferase-1 (PRMT-1), which preferentially associate with SRC-1 over SRC-2 or SRC-3 (138,139). In addition to the basic complex of SRCs, CBP/p300, HATs, and methyltransferases bound to liganded ER on the ERE/promoter region of estrogen-regulated

genes, a host of other coactivators are recruited to the complex to enhance ER transcriptional activity. Combining what is known about coactivator complex formation, we can hypothesize a model of coactivator recruitment in which the agonist-bound ER binds to ERE sites in the promoter of responsive genes and recruits SRC coactivators to the ER-LBD. SRC interacts with the coactivators SRA (steroid receptor RNA activator) and p68/72, which are recruited to the ER AF-1 domain (146,147). Interactions between these AF-1 coactivators and SRC help stabilize the coactivator complex and synergize interactions between the AF-1 and AF-2 domains of ER. In addition, SRC coactivators recruit HAT coactivators CBP/p300, p/CAF, histone methyltransferase coactivators such as CARM-1 and PRMT-1, and other chromatin remodeling coactivators through the SWI/SNF complex (51,148–150). In an inactive state, DNA is tightly bound to histones and compacted in chromatin. The tightly packed chromatin lends structural stability but restricts the accessibility of promoters, ERE, and general transcriptional machinery, adding a layer of complexity to ER gene activation. The CBP/p300, p/CAF, CARM-1, PRMT-1, and SWI/SNF coactivators remodel the packed chromatin, thus enhancing accessibility to the promoter region and facilitating RNA pol II recruitment. The final group of coactivators recruited to the ER/coactivator complex includes TRAP220 (thyroid receptor-associated proteins) and DRIP (vitamin D receptor-interacting proteins); the TRAP/DRIP complex has been indicated in directly connecting the ER/coactivator complex to basal transcriptional machinery (151–153). Each coactivator serves to enhance the ability of ER to activate transcription of responsive genes. The intricate recruitment and combination of these and other coactivators to the agonist-bound ER has been shown to be affected by receptor profile of the cell, type of ligand bound to the receptor, cell type, tissue type, and drug treatment (99,154–156). Therefore, specific coactivator repression is being extensively studied as a viable option for reducing ER signaling in breast cancer cells.

An opposing group of coregulators, called corepressors, interacts with ER to inhibit transcription of target genes (Table 2). Corepressors such as SMRTs and NCoR (Fig. 4) interact with unliganded ER (157,158) and also in the presence of ER antagonists such as tamoxifen and RU486 (159,160). The ER-associated corepressors SMRT/NCoR then recruit other corepressors such as HDAC to inhibit transcriptional activation by ER. Next, other corepressors are recruited to the complex, including SHP (short heterodimer partner), an orphan receptor that binds the NR box in the LBD of ER- $\alpha$  and ER- $\beta$  in a ligand-dependent manner to antagonize the binding of SRC coactivators (161–163), MTA-1 (metastases-associated protein-1) with HDAC recruitment capabilities (164–166), and MAT-1 (menage a trios 1) that interacts with general transcriptional machinery to repress ER-mediated transcriptional activity (164–166) (Fig. 5). A recently identified corepressor, LCoR (ligand-dependent corepressor), has been shown to bind to the ER- $\alpha$  NR box in a ligand-dependent manner to block SRC binding and recruit HDAC (167). The significance of corepressor activity to ER signaling has been explored in experiments that have shown that HDAC activity and corepressor recruitment are required for the transcriptional repression of tamoxifen-bound ER- $\alpha$  (107,168). In addition, it has been reported that decreased amounts of NCoR correlate with acquisition of hormone resistance in a mouse model of breast cancer (168). The general mechanism by which corepressors exert their inhibitory effects is to bind the unliganded ER and obstruct the ligand-independent recruitment of coactivators. In light of the fact that unliganded ER is still able to bind ERE sites, it

**Table 2**  
**Corepressors and Their Function**

CoRepressor	ER domain binds	Function	Reference
DAX-1	AF-2	Orphan receptor that inhibits ER activation by occupying the ligand-induced coactivator-binding surface and recruiting corepressors	(328)
LCoR	LBD	HDAC-dependent and -independent repression of ligand-bound ER; recruits C-terminal binding corepressors to ER	(167)
MAT-1	AF-2	Recruits CDK-activating-kinase and interacts with TFIIF general transcription factor to block ER transactivation	(164)
MTA-1	LBD	Represses ER-activated transcription by recruiting HDACs to ERE sites; inhibits phosphorylation of ER; interacts with MAT-1 protein; variant of MTA-1 has been found that sequesters ER in cytoplasm	(164–166)
NcoR (RIP-13)	Hinge region	Silences basal transcription by directly interacting with transcriptional machinery; represses partial agonist activity	(80,168,329)
REA	LBD	Represses estrogenic activity and enhances antiestrogenic activity; competes with ER for binding to SRC-1 and other coactivators	(330,331)
RTA	AF-1	Contains an RNA recognition motif; expression in cells inhibits TAM-mediated partial agonist activity and partially inhibits estrogen-mediated transcription	(332)
SHP	LBD	Interacts with the NR box of ER- $\alpha$ and ER- $\beta$ in a ligand-dependent manner to repress transactivational activity; may block binding of SRC coactivators to NR box	(161–163)
SMRT	LBD	Silences basal transcription; interacts with HDAC1; blocks antiestrogenic/partial agonist transcriptional activation of ER	(80,159,168)
Ssn3	AF-1	Blocks transcriptional activity induced by both agonists and partial antagonists	(43)

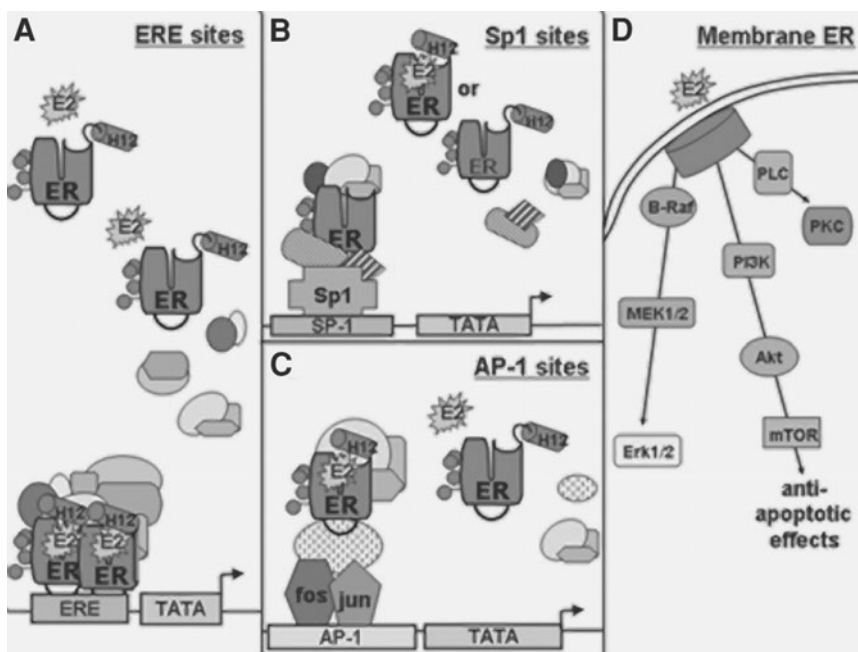


Fig. 6. Multiple mechanisms by which ER activates a variety of responsive genes. (A) Representation of classic pathway of estradiol-dependent ER-mediated transcription of estrogen-responsive genes through ERE elements; (B) nonclassic tethered hormone-dependent and hormone-independent interactions between ER and Sp1 sites in promoter region of Sp1-responsive genes; (C) nonclassic tethered hormone-dependent activation of genes whose promoters contain AP-1 elements through interactions between ER and fos/jun family of transcription factors; (D) rapid nongenomic hormone-dependent ER activation of various cell signaling pathways.

has been hypothesized that the true manner in which corepressors inhibit ER-responsive gene activation is by blocking ligand-independent transactivation pathways that impinge on the ER.

### **ERE-Independent Genomic Actions of ER**

In addition to ER/ERE-mediated activation of estrogen-responsive genes, on binding estradiol, ER- $\alpha$  and ER- $\beta$  can activate the expression of a multitude of genes that are not regulated by ERE sites. To activate non-ERE-regulated genes, estradiol-bound ER either directly binds DNA sequence-specific elements within the promoter region of these genes, or indirectly contacts these alternate response elements through interactions with mediator proteins that tether the ER to the promoter. For example, both ER- $\alpha$  and ER- $\beta$  can activate transcription of genes, such as insulin-like growth factor (IGF-1) and collagenase, through AP-1 elements in these genes' promoter regions (Fig. 6) (48). AP-1 activation occurs when the N-terminus of ER interacts with the Fos/Jun transcription factor complex on AP-1 sites (34,37,49). ER- $\alpha$  and ER- $\beta$  both activate transcription from AP-1 sites after ligand binding, but the ERs respond in an opposite manner to typical ER agonists and antagonists. In response to agonists such



as estradiol and DES or the SERM tamoxifen, ER- $\alpha$  activates AP-1 sites. Conversely, ER- $\beta$  activates AP-1 sites after binding tamoxifen or raloxifene, but estradiol binding antagonizes ER- $\beta$  AP-1 activation (34). The differential response of ER- $\alpha$  and ER- $\beta$  to these ligands is thought to be due to differences in the AF-1 and AF-2 domains of the two ERs, or to disparities in coactivator recruitment (37,48).

Another example of non-ERE-mediated ER activation of target genes occurs through the tethered complex of ligand-bound ER- $\alpha$  and the Sp1 transcription factor at GC-rich promoter sequences (Fig. 6) (169–172). Both ER- $\alpha$  and ER- $\beta$  activate transcription of genes with GC-rich Sp1 promoter sites, such as the *retinoic acid receptor  $\alpha$ 1* (*RAR $\alpha$ 1*) gene, by forming a tethered complex of ER bound to Sp1 at the promoter site (173,174). Another example of enhancing target-gene expression is the tethered interaction of ER- $\alpha$  with the c-rel subunit of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) complex (175). ER binding to the NF- $\kappa$ B complex inhibits NF- $\kappa$ B-mediated interleukin-6 gene expression (175,176). Recent evidence has emerged that chemotherapy drugs, which specifically block NF- $\kappa$ B activation, cause apoptosis and regression of breast cancer in mice (177). Other studies have focused on ER- $\alpha$  interactions with the NF- $\kappa$ B complex as possible therapeutic targets for treating ER-negative breast cancer (178).

Estrogen-dependent signaling to ER results not only in ER translocation to the nucleus to directly or indirectly bind DNA transcriptional elements, but also in ligand-bound ER translocation to the plasma membrane (Fig. 6). As early as 1977, researchers hypothesized the existence of a membrane-associated ER to explain rapid cellular responses (cAMP generation and Ca<sup>2+</sup> flux) to estradiol (179–182). In endothelial cells, researchers have shown that estradiol-mediated membrane effects cause activation of the ras, raf, MAPK, and Erk phosphorylation cascade (183). Other experiments using endothelial cells have shown that estradiol membrane effects activate the phosphatidyl inositol 3' icinase (PI3K)/Akt signaling cascade resulting in inhibition of apoptosis (184). A membrane ER has been hypothesized to explain rapid estradiol-mediated membrane effects that lead to protein kinase C (PKC) activation (185,186). Researchers suggest that estradiol membrane effects occur as quickly as two minutes after estradiol exposure, too rapid an effect to be mediated by transcription of estrogen-responsive genes, and can be blocked by specific ER antagonists such as ICI 182,780 (182,184,187). Researchers seeking the exact location of a membrane ER have used antibodies to ER- $\alpha$  and located endogenous membrane ER in a number of cell types (188–190). Translocation of ER- $\alpha$  or ER- $\beta$  from the nucleus to the plasma membrane has been illustrated by experiments in which a single cDNA for ER was expressed in cells resulting in not only a nuclear pool of ER, but also a pool of membrane ER (191). The cellular-signaling implications of a membrane ER that influences multiple phosphorylation signaling pathways presents widespread opportunities for estrogen and ERs. Each of the alternative signaling pathways—AP-1, Sp1, NF- $\kappa$ B, and membrane ER—expand our knowledge about ER-mediated gene activation and contribute to future advances in breast cancer treatment.

### **Ligand-Independent Activation of ER**

In the absence of estradiol, ligand-independent activation of ER occurs primarily through signaling pathways downstream of growth factors that activate ER by enhancing phosphorylation of various Ser and Thr residues in the AF-1 and AF-2 domains of ER (Fig. 7). Increased phosphorylation of ER influences receptor dimerization, coactivator and corepressor recruitment, and the effects of agonists and

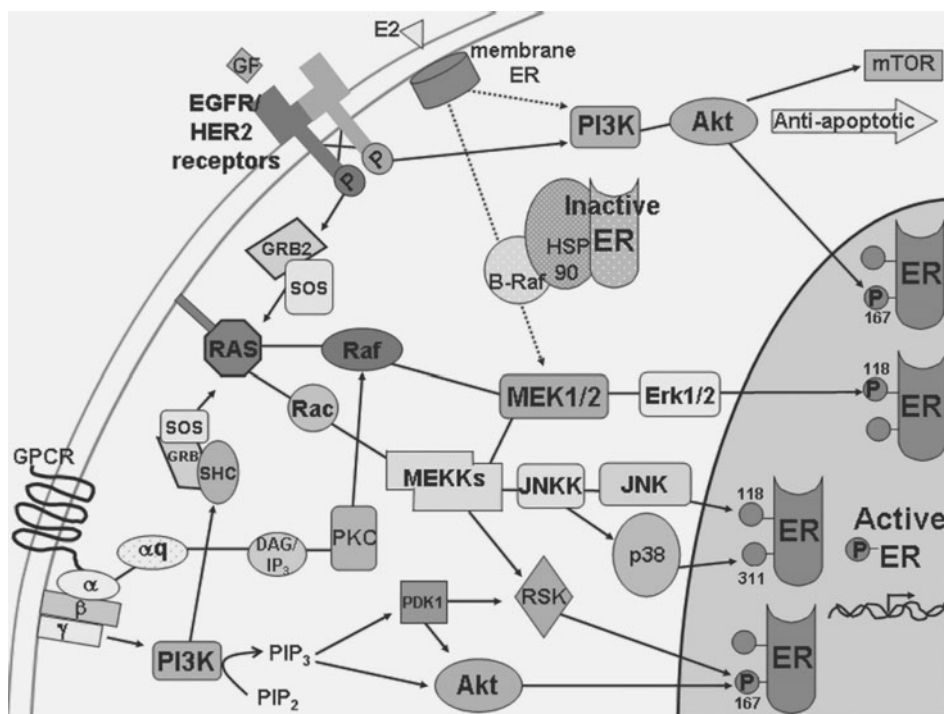


Fig. 7. Cross talk and signaling with ER. Signaling from multiple growth factor signaling pathways, as well as G-protein-coupled receptors, impinge on ER-mediated signaling and transactivation in a nongenomic manner.

antagonists on ER-dependent gene activation. Therefore, signaling exchange that alters the phosphorylation status of ER affects every level of ER influence in the cell. Interaction between various cellular-signaling pathways is commonly referred to as “cross talk.” To date, many signaling pathways have been shown to cross talk with ER, including signaling pathways downstream of growth factor receptors such as EGF/EGFR (54,55,192), factors that regulate cellular phosphorylation levels such as PKA and PKC (55,193,194), IGF/insulin receptor (195–197), and transforming growth factor- $\beta$  (TGF- $\beta$ ) (198) (Fig. 7). In addition, many factors, other than estradiol signaling, have been shown to influence the activation of ER, including dopamine (199), cAMP (193,194,199,200), insulin and IGF-1 (54,193,201,202), heregulin (203,204), and TGF- $\alpha$  (198,205).

Growth-factor signaling represents the largest class of ER activators. The first report of cross talk involving ER and another signaling pathway arose from experiments demonstrating that EGF mimicked estrogen-stimulated growth in the mouse uterus, and antibodies to EGF or treatment with a pure antiestrogen inhibited this response (192,206). The importance of ER-EGFR cross talk was illustrated *in vivo* when mice engineered to lack ER- $\alpha$  (ERKO mice) showed no such uterine response to EGF treatment (207). The mechanism of EGFR-ER cross talk is EGF signals to EGFR triggering a phosphorylation cascade through MAPK and Erk resulting in the phosphorylation of Ser118 of the ER- $\alpha$  AF-1 domain and ER transactivation (54,55,206). Various

tyrosine kinase receptors are able to signal either by the ras, raf, MAPK cascade or by the PI3K, AKT cascade to effectively phosphorylate Ser118 and Ser167 and activate ER- $\alpha$  (208). The MAPK pathway is central to signaling cross talk with ER. In addition to mediating the phosphorylation of ER- $\alpha$  Ser118, *in vivo* and *in vitro* evidence has shown that a kinase downstream of MAPK signaling, RSK (p90 ribosomal S6 kinase), is responsible for phosphorylating Ser167 (60,63).

Cross talk with ER is mediated by cell-cycle-regulating proteins such as cyclin-dependent kinases (CDK), cyclin A, and cyclin D1. Experimental evidence has shown that the CDK2/cyclin A complex phosphorylates Ser104 and Ser106 and, thus, increases estrogen-dependent and estrogen-independent transactivation of ER- $\alpha$  (209). Additionally, cyclin D1 plays an important role in the development of normal mammary gland and the proliferation of mammary epithelium during pregnancy (210,211). Transgenic mice overexpressing cyclin D1 are known to be predisposed to mammary carcinoma (212), and cyclin D1 is overexpressed in some breast tumors (213–215). Cyclin D1 can bind to the LBD of ER- $\alpha$  directly and has been found to activate ER- $\alpha$  independent of estradiol and potentiate the transcriptional activity of the estradiol-ER complex (215,216).

Heregulin is known to promote hormone-independent human breast cancer cell growth (203). In addition, overexpression of the receptor for heregulin, HER-2, confers hormone-independent growth properties to breast cancer cells, resulting in cells that are insensitive to estradiol and tamoxifen (203,204). One mechanism by which heregulin may influence the activity of ER is through phosphorylation. Experiments in breast cancer cells have shown that heregulin activation of HER-2 results in direct and rapid phosphorylation of tyrosine residues of ER and ER transactivation of the PR gene (119,204). Future studies to elucidate the lines of cross talk between ER and multiple cellular-signaling pathways may provide useful clues to the initiation and progression of breast cancer.

## Endocrine Therapy and Treatment of Breast Cancer

Experimental studies spanning the past 100 yr have sought to determine what factors cause the initiation and progression of breast cancer. From Beatson's 1896 study (4) demonstrating improved outcome after removal of a premenopausal breast cancer patient's ovaries to the latest data on the efficacy of aromatase inhibitors (217), 17 $\beta$ -estradiol has proven to be the overriding factor that influences every stage of breast cancer. Since the hormone estradiol plays a central role in breast cancer, it is not surprising that ERs are also key targets for investigation and pharmaceutical manipulation. As pointed out in this chapter, estradiol signaling with ER is not a simple bipartite system. Rather, multiple cellular-signaling pathways and many target genes regulated by a variety of DNA elements culminate in an overall estrogen-signaling burden (119). Because overall estrogen exposure is a critical risk factor for development of breast cancer, drugs developed to prevent or inhibit this disease have been designed to reduce estrogen signaling by targeting multiple members of the complex signaling system. A number of such compounds used clinically have been evaluated to treat or prevent breast cancer (218). Approx 70–80% of all breast tumors express ER (ER positive) (30,219), and, therefore, drugs that are currently standard for treating breast cancer are endocrine therapies that target estrogen action either through blocking ER function or by inhibiting estradiol synthesis. For ER-positive premenopausal women, endocrine treatments include tamoxifen, ovarian ablation/suppression, or a combination of the

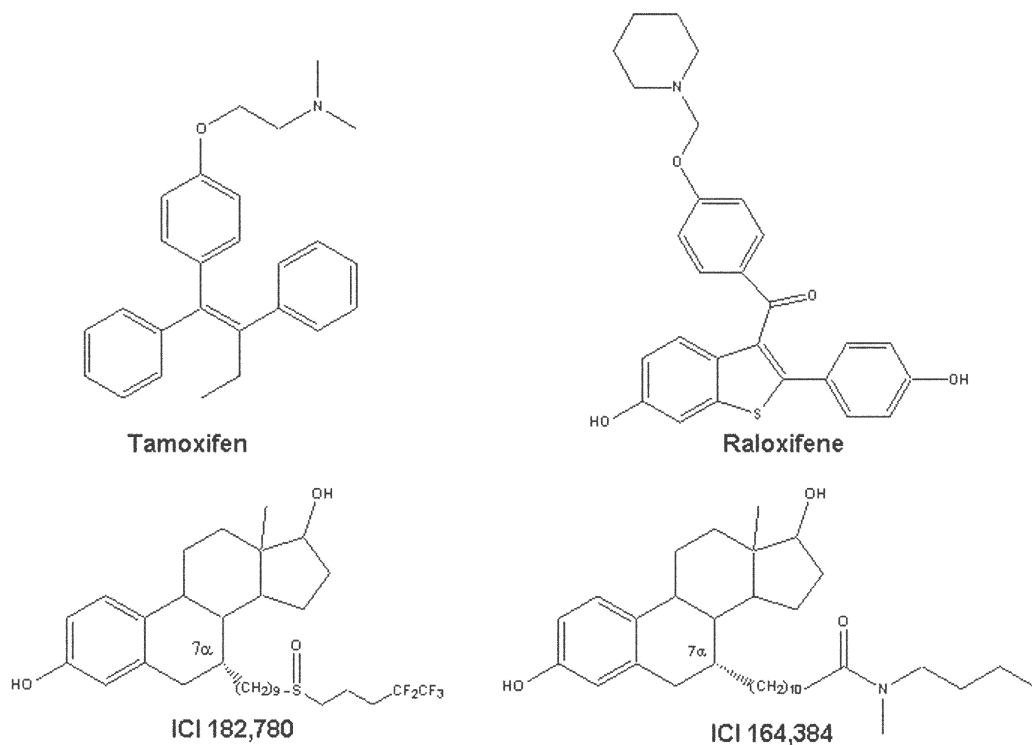


Fig. 8. Chemical structures of SERMs and pure antiestrogens. Compounds that affect breast cancer: SERMs including tamoxifen and raloxifene and pure antiestrogens including ICI 182,780 and ICI 164,384.

two (218,220–222). For ER-positive postmenopausal women, tamoxifen has been the preferred option for 15 yr, but ongoing studies of the efficacy of aromatase inhibitors, such as anastrozole and letrozole, are proving that the third-generation aromatase inhibitors are more effective with fewer side effects (223–225).

### ***Tamoxifen as an Antitumor Agent***

Tamoxifen, a nonsteroidal antiestrogen, is converted to the active metabolite 4-OH tamoxifen, which competes with estradiol for ER- $\alpha$  binding (226) (Fig. 8). Tamoxifen-bound ER- $\alpha$  still binds to ERE sites, but tamoxifen binding triggers a different conformational arrangement of ER H12 than that induced by estradiol-ER binding (78,227, 228). The alkylaminoethoxy phenyl side chain of tamoxifen prevents H12 from enveloping the ligand in the hydrophobic binding pocket of the ER LBD, and this antagonist conformation of H12 obscures the coactivator binding site of ER, inhibiting coactivator recruitment that is essential for full estradiol-induced ER transactivation activity (130,229,230).

Tamoxifen is currently used as a 5-yr adjuvant treatment for patients with ER-positive breast cancer (231). Overall, 5 yr of tamoxifen reduces the incidence of contralateral breast cancer by approx 50% and produces a survival advantage in patients with node-positive or node-negative breast cancer (231,232). However, tamoxifen cannot

be viewed as a cure for breast cancer because a portion of breast tumors predicted to respond do not, and both laboratory and clinical data have shown that, after 5 yr of treatment, initially responsive tumors may become tamoxifen resistant (233–235).

### ***Tamoxifen Resistance and Tamoxifen-Stimulated Breast Tumors***

Insights into future SERM design as well as a better understanding of tamoxifen's actions in specific tissues could be gained from determining the mechanism by which resistant breast cancer cells escape the antagonistic signaling of tamoxifen. Multiple mechanisms have been proposed for tamoxifen resistance, including estrogen availability and local metabolism of tamoxifen, loss and/or mutation of ER, alternative signal transduction pathways, and agonist/antagonist balance of tamoxifen. The description of tamoxifen-stimulated tumor growth is derived from laboratory studies (234) and from anecdotal clinical reports (233,236), which showed that tamoxifen withdrawal can inhibit the growth of breast and endometrial tumors that had begun to recognize tamoxifen as an agonist and not an antagonist (231,237,238).

### ***Estrogen Availability and Tamoxifen***

Tamoxifen is reported to cause an increase in circulating estradiol levels in premenopausal women (239–242). Because tamoxifen and estradiol compete with each other for ER binding, a high concentration of estradiol could potentially block the antiestrogen effects of tamoxifen on breast cancer. This hypothesis is supported by the clinical observation that patients with stage IV disease who initially respond to tamoxifen and subsequently develop tamoxifen resistance still derive benefits from estradiol ablation and ovariectomy (243).

In an effort to identify the possible source of estrogenic or tumor-promoting stimuli that may lead to tamoxifen-resistant breast tumors after long-term treatment, the pharmacokinetics and metabolism of tamoxifen have been extensively studied in patients (244–246). Previous studies have hypothesized that long-term tamoxifen treatment may lead to appreciable sera concentrations of the more potently estrogenic tamoxifen metabolite *E* isomer (tamoxifen without dimethylaminoethane side chain), and that this *E* isomer is the agent causing the observed tamoxifen-stimulated tumor growth (247–249). This hypothesis has been refuted by experiments in which a fixed-ring version of tamoxifen, incapable of metabolizing to the *E* isomer, is capable of supporting the growth of tamoxifen-dependent tumors (250). Therefore, no evidence exists for reduced absorption, distribution, or increased concentrations of estrogenic metabolites in sera produced by long-term tamoxifen treatment (246). Nevertheless, patients with ER-positive breast cancer treated long term with tamoxifen show signs of tamoxifen resistance and estrogen sensitivity that must be treated for successful inhibition of breast cancer.

Tamoxifen is an established adjuvant therapy for postmenopausal women with ER-positive breast cancer. In addition to issues of tamoxifen resistance after long-term treatment, tamoxifen acts as an agonist in the uterus causing an increase in endometrial cancer and thromboembolic disorders (238). A category of drugs called aromatase inhibitors block the aromatase enzyme responsible for converting testosterone to estradiol, thereby reducing circulating amounts of estradiol, estrone, and estrone sulfate (3,251). Results from randomized trials comparing tamoxifen to aromatase inhibitors such as anastrozole and letrozole or the aromatase inactivator exemestane as treatments for metastatic breast cancer have shown that both anastrozole and letrozole had equivalent or improved efficacy compared with tamoxifen, with similar or decreased toxicity (Fig. 9) (225,252–

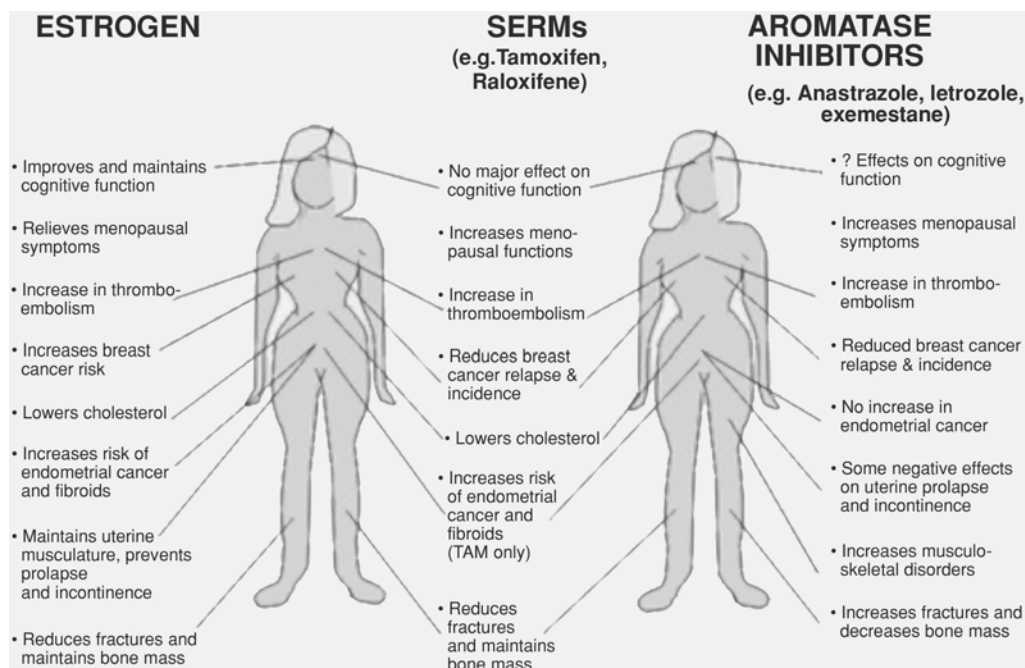


Fig. 9. Contrasting actions of estrogen, SERMs, and aromatase inhibitors.

254). These studies have led to the approval of both anastrozole and letrozole as endocrine therapy for postmenopausal women with metastatic breast cancer. While compelling, these studies were of women with metastatic breast cancer who received anastrozole for an average of approx 1 yr (225,255), and are not useful for determining possible side effects associated with a 5-yr course of aromatase inhibitors. Therefore, the ATAC (anastrozole alone or in combination with tamoxifen) trial has been established to compare the long-term safety and efficacy of tamoxifen, the aromatase inhibitor anastrozole, or a combination of both as an adjuvant therapy for postmenopausal women with early ER-positive breast cancer (217).

The first results from the ATAC trial stated that anastrozole is an effective and well-tolerated endocrine therapy for postmenopausal women with ER-positive breast cancer, but until full long-term results are reviewed, a 5-yr course of tamoxifen treatment is still the best adjuvant endocrine therapy (Fig. 9). The results of the ATAC trial are based on data from the first 33 mo in which approx 3000 women per cohort of the study received either tamoxifen alone, anastrozole alone, or both tamoxifen and anastrozole. After 33 mo, the ATAC study showed no difference in overall survival among any of the groups. In addition, although the overall disease-free survival is statistically significantly greater for the anastrozole group (90%) than the tamoxifen group (88%), the absolute difference in percentage of patients disease free at follow-up is only 2% (217). At the time of reporting, the only short-term side effects not shared with tamoxifen and exhibited only in the anastrozole treatment group were increased musculoskeletal disorders and bone fractures (217). In conclusion, the ATAC trialists' group pointed out that the evaluation of a 5-yr course of anastrozole and tamoxifen

treatment is still required to see the full benefits of either treatment. The American Society of Clinical Oncology (ASCO) assessment of the ATAC trial concludes that, although tamoxifen is not devoid of side effects, there are extensive long-term data on patients and a clearer understanding of risks, and, therefore tamoxifen remains the adjuvant endocrine therapy of choice for patients with early-stage breast cancer (225).

Aromatase inhibitors such as anastrozole are not appropriate treatments for premenopausal women who have ovarian function because inhibition of aromatase will lead to a feedback cycle in which the ovaries become overactive to combat anastrozole's effects (256). Ovarian ablation has been successfully used to treat breast cancer in premenopausal women and is effective as an adjuvant therapy to increase disease-free survival, as demonstrated by the Early Breast Cancer Trialists' Collaborative Group clinical trials (257). Ovarian ablation by ovariectomy and irradiation is associated with long-term adverse effects, such as osteoporosis and increased cardiovascular problems (258). A class of drugs called luteinizing hormone–releasing hormone (LHRH) agonists (goserelin, leuporelin) is available to suppress estradiol concentrations to postmenopausal levels, and these ovarian ablation effects are reversible after ceasing treatment. Results from the Zoladex Early Breast Cancer Research Association trial comparing the efficacy and safety of goserelin vs the chemotherapy regimen of cyclophosphamide/methotrexate/5-fluorouracil (CMF) concluded that goserelin provides equivalent efficacy without the cytotoxic side effects of CMF in premenopausal women with ER-positive breast cancer (259). In addition, other clinical trials comparing goserelin and tamoxifen for treatment of node-positive, ER-positive breast tumors in premenopausal women have shown no differences in recurrence or overall survival between the treatment groups (221). The most recent large randomized clinical trial by the Austrian Breast and Colorectal Cancer Study Group compared the efficacy and safety of either a combination of goserelin and tamoxifen or CMF for treatment of ER-positive breast tumors in premenopausal women (222). In this study, of more than 1000 women treated with goserelin for 3 yr plus tamoxifen for 5 yr or 6 cycles of CMF chemotherapy, 17.2% of the goserelin/tamoxifen group and 20.8% of the chemotherapy group showed recurrence of breast cancer (222). Based on 5 yr of follow-up data, researchers concluded that the goserelin/tamoxifen combination was significantly more effective than CMF in the adjuvant treatment of premenopausal women with stage I and II breast cancer (222).

Taken together, the results from these various clinical trials for postmenopausal and premenopausal women with ER-positive breast cancer indicate that estrogen inhibition by either aromatase inhibitors or temporary ovarian ablation in combination with tamoxifen, respectively, are effective antiestrogenic adjuvant breast cancer treatments.

### **Loss or Mutation of ER- $\alpha$**

For breast tumors that are ER-negative, the endocrine treatment possibilities are considered to be inappropriate. In addition, it is thought that breast tumors can progress from ER-positive status to ER-negative status over time, with ER-negative status conferring a much worse prognosis (129,260). Because most ER- $\alpha$ -negative breast tumors do not respond to tamoxifen therapy and some advanced tumors become ER- $\alpha$  negative (261), it was hypothesized that the loss of ER- $\alpha$  may be one mechanism of tamoxifen resistance. This hypothesis has been refuted as additional clinical studies have shown that breast tumors that eventually fail tamoxifen therapy remain ER-positive and laboratory models of tamoxifen resistance also remain ER-positive (262–265).

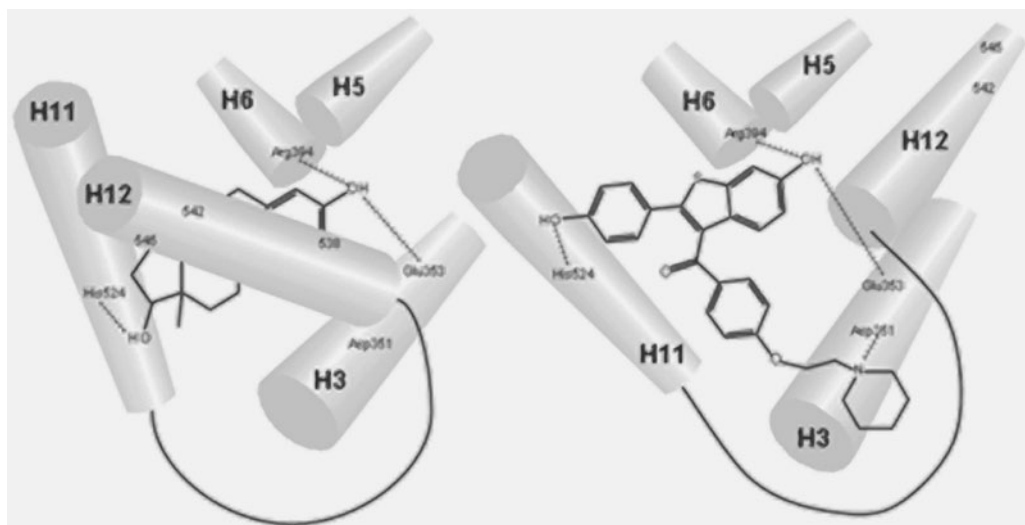


Fig. 10. Schematic representation of ER- $\alpha$  bound with estradiol or raloxifene. (A) Estradiol binding to ER- $\alpha$  induces conformational changes causing helix 12 to form a lid covering the occupied LBD. (B) Raloxifene binding to ER- $\alpha$  induces conformational changes dissociating helix 12 from contact with the LBD and masks the coactivator recruitment regions of ER- $\alpha$ .

Small alterations in the genetic or protein structure of ER- $\alpha$  have been found to affect cellular responsiveness to tamoxifen (117). For example, on examination of 20 tamoxifen-sensitive and 20 tamoxifen-resistant breast tumors using single-strand conformation polymorphism analysis, Karnik et al. (266) noted that 10% of the tamoxifen-resistant tumors harbored mutations in exon 6, which encodes part of the ER- $\alpha$  LBD. Furthermore, while screening tamoxifen-stimulated MCF-7 breast tumors, a single point mutation was identified at amino acid 351, aspartic acid to tyrosine (D351Y), in the LBD of ER- $\alpha$  (92). The D351Y ER- $\alpha$  compared with wild-type ER- $\alpha$  responds with increased activity to a tamoxifen analog (110) and responds to the normally antiestrogenic pharmacology of raloxifene as a partial agonist (267,268). The crystal structure of raloxifene-occupied ER- $\alpha$  has shown that raloxifene directly contacts ER at aspartic acid 351 by hydrogen bonding with the long antiestrogenic side chain of raloxifene (Fig. 10). These data, combined with recent findings in which a raloxifene derivative (R1h) without its antiestrogenic side chain piperidine ring was a potent agonist for wild-type ER- $\alpha$ , have led to the conclusion that raloxifene exerts an antiestrogenic effect on ER- $\alpha$  by shielding the critical D351 amino acid (94). Clearly, the study of ER- $\alpha$  mutations found in tamoxifen-stimulated cells not only may provide useful insights into mechanisms of drug resistance but also may show how ERs interact with a variety of ligands and SERMs.

### **Agonist/Antagonist Balance of SERMs**

The SERMs tamoxifen and raloxifene (Fig. 8) both bind ER and have agonist activity in certain *in vitro* breast cancer cell models and in certain estrogen-responsive tissues such as uterus and bone (40,129,267,269). The tissue-specific agonist and



antagonist effects of SERMs suggest that the specific factors controlling the actions of these SERM may be differentially expressed in various cells and tissues. To date, numerous coactivators and corepressors of ER function have been implicated as mediators of tissue-specific responses to SERMs. In addition, evidence from cocrystal structures of ER LBD complexed with various SERMs has provided additional insight into the mixed actions of SERMs.

Coactivator and corepressor recruitment to the ER dictates the activation status of estrogen-responsive genes within a cell (168,270). Experimental evidence has shown differential recruitment of coactivators and corepressors to the ER-tamoxifen complex compared to the ER-raloxifene complex in two tissue types: breast and endometrium (271). In breast cancer cells, where tamoxifen and raloxifene are both antagonists, the binding of either SERM to the ER resulted in the recruitment of corepressors, not coactivators, to ER target gene promoters (271). In endometrial cancer cells, where tamoxifen is an agonist and raloxifene is an antagonist, only tamoxifen binding to ER resulted in coactivator recruitment to ER target genes (271). In particular, SRC-1 was found to be required for the agonist actions of tamoxifen in endometrial cells. These data describe how the particular set of coactivators and corepressors, and the concentration at which each is present in a cell type, are directly related to the agonist or antagonist activities of the SERM-ER complex.

Another factor influencing the specific actions of SERMs bound to ER is the impact that a particular ligand may have on subsequent ER stability and degradation. While it has been well established that estradiol promotes the degradation of ER (272), SERMs such as tamoxifen increase steady-state levels of ER- $\alpha$  (273,274). Experiments have suggested that coactivator binding to the AF-2 domain of estradiol-ER may promote ER- $\alpha$  degradation and that lack of coactivator binding prevents ER degradation (275,276). Tamoxifen binding to ER causes a well-characterized "antagonist" conformation of H12 that masks the SERM-bound ER's coactivator recruitment site, therefore preventing coactivator binding and repressing the transcriptional activation of ER. In addition to the antagonist effects tamoxifen causes by blocking coactivator binding to ER, it is possible that tamoxifen also may cause agonist effects by preventing ER degradation in some cells (270,277). A well-studied example of an antagonist that affects ER stability is the pure antiestrogen ICI 182,780 (Fig. 8), which has been shown to promote ER degradation as its primary mode of ER antagonism (276). New insights into how ICI 182,780 and other pure antiestrogens may affect ER degradation have been gained with the study of the 3D crystal structure of ER- $\beta$  bound to ICI 164,384 (Fig. 8) (278). The study showed that ICI 164,384 binding to ER- $\beta$  occurred in much the same manner as tamoxifen or raloxifene binding, but the conformation of ER's H12 induced by ICI 164,384 binding was very unlike the simple antagonist conformation induced by the binding of tamoxifen and raloxifene. The ER-LBD 11 $\beta$  channel accommodates the bulky side chain of SERMs and ICI 164,384 (86,87), but the size and positioning of the ICI side chain allows it to closely align with ER's AF-2 region, causing H12 to adopt a unique conformation. While tamoxifen induces a well-characterized antagonist conformation of H12 that blocks coactivator recruitment to ER, this conformation still allows some H12-LBD contact that may result in partial agonist activity (86,87). Alternatively, the pure antiestrogen ICI 164,384 completely abolishes any association between H12 and the ER-LBD, leaving a large exposed hydrophobic LBD surface that may facilitate corepressor recruitment (278). The novel ER-H12 conformation induced by ICI 164,384 resembles a misfolded or denatured protein (278). This

type of protein may then be targeted by the cell for degradation, thus providing a mechanism for experimental observations that ICI 164,384 binding causes rapid degradation of ER (279). The pure antiestrogens, exemplified by ICI 164,384 and ICI 182,780 provide an interesting example of antiestrogen action by altering receptor stability, which results in the rapid degradation of the ER complex (280).

## Future Perspectives

### *Molecular Biology*

Estradiol and various SERMs affect ER signaling by a specific sequence of events. The ligands induce distinct ER structural conformations that ultimately result in binding of the complex to specific estrogen-response sites on DNA. Additionally, ligand binding affects the stability and degradation of the ER, influences the cross talk between ER and other signaling pathways, and dictates the recruitment of coactivators and corepressors. Any or all of these factors may be the key to predicting the particular agonist and antagonist effects that a SERM or novel compound may have in a specific tissue or cell type. Therefore, each of these mechanisms of action must be thoroughly investigated in the future using genomic and proteomic analyses. Molecular techniques such as DNA microarray and real-time polymerase chain reaction can be used to quantify the positive and negative effects of a drug or compound on the transcription of a particular gene or family of genes (281). The field of proteomics has the potential to be extremely versatile. It is possible to identify signaling proteins and interpret the complex cross talk impinging on the ER (282) and, at the same time, screen for serum biomarkers useful in the early detection of breast cancer (283). Using chromatin immunoprecipitation assay techniques, it is now possible to choose specific DNA-promoter elements and analyze the specific cohort of coactivators bound to ER in a particular cell compared with another cell type, or before treatment with a SERM and after treatment (270,271). In addition, with advances in X-ray crystallography, researchers will soon be able to analyze the crystal structure of the entire ER- $\alpha$  protein bound to estradiol and compare this with ER bound to pure antiestrogens and various SERMs. Analysis of the whole ER- $\alpha$  protein bound to agonists and antagonists will provide invaluable insight into how interactions between the DBD and other domains such as the AF-1 domain influence ER function.

Tissue microarrays now allow high-throughput molecular profiling of breast cancer specimens, which will be essential for the analysis of prognostic gene status (e.g., *ER*, *PR*, *Her-2/neu*) as well as in the search for novel diagnostic biomarkers (284). By way of example, it will soon be possible to integrate molecular-detection techniques to compare gene, protein, and tissue biomarker alterations in breast tumors as they progress from tamoxifen-responsive to tamoxifen-resistant and tamoxifen-stimulated growth to determine the mechanisms of breast tumor progression. Similarly, by targeting specific members of cellular signaling pathways known to influence estrogen signaling through cross talk, endocrine therapy and even chemotherapy can more accurately pinpoint cellular targets to inhibit breast cancer growth (218). For example, recent advances have led to the development of chemotherapy that is able to specifically target the NF- $\kappa$ B cellular signaling pathway in breast cancer cells (177,178). With further analysis of cross talk between cellular signaling pathways and ER, perhaps drugs can be developed to successfully stimulate a breast cancer cell's existing apoptotic machinery with novel therapeutic agents.

### **Clinical Applications**

During the past 30 yr, enormous changes have occurred in the strategic application of antiestrogens. Originally their action as antifertility agents in animals held the promise of an application as birth control or “morning-after” pills. Studies in the 1960s demonstrated that the drugs, in fact, induced ovulation, so the pharmaceutical goal changed to a therapy for treating infertility (285). With the discovery of the ER and the knowledge that estrogen controlled breast tumor growth, the search for possible applications of antiestrogens shifted to breast cancer treatment (286,287). After more than 20 yr of clinical use, tamoxifen became the gold standard for the treatment of all stages of breast cancer during the late 1990s. Millions of women have been prescribed tamoxifen because of the unequivocal observation that the drug confers numerous advantages with few side effects.

The finding in 1987 that antiestrogens maintain bone density (288,289) completely changed the prospects for the prevention of breast cancer in postmenopausal women. Tamoxifen and raloxifene both maintain bone density, prevent mammary carcinogenesis, and have antiuterotropic activity, although raloxifene is less estrogenic in the uterus (288,290). Clearly, an application to prevent osteoporosis could have the advantage of preventing breast and endometrial cancer as a beneficial side effect (291). Two ongoing studies monitoring breast cancer as a primary or coprimary end point, the Raloxifene for Use in The Heart trial (292) and the Study of Tamoxifen and Raloxifene trial, should have definitive data by 2005 (293–295). Confirmation of the hypothesis that targeted antiestrogenic drugs can prevent breast cancer will not only establish the topic as a textbook example of successful translational research but also lead to a revolution in women’s health.

Recent surprising findings from the Women’s Health Initiative trial, which aimed to assess the benefits and risks of hormone replacement therapy (HRT) for postmenopausal women, showed that HRT increased the incidence of heart attack (29%), stroke (41%), coronary vascular disease (22%), and even breast cancer (26%), with the only positive effects of HRT seen as a reduction in some types of fractures and colorectal cancer (36%) (296). The results illustrate that new approaches to hormone replacement are necessary to target specific diseases for prevention. Future efforts must aim to prevent menopausal symptoms including hot flashes without increasing health risks. Clearly, enormous opportunities exist for the pharmaceutical industry to target estrogen signaling in a tissue- and cell-specific context.

An increasing array of agents is currently undergoing clinical evaluation for breast cancer prevention and treatment, including novel SERMs, aromatase inhibitors/inactivators, LHRH agonists, inhibitors of tyrosine kinase receptors, and cyclooxygenase-2 inhibitors (218,297). For example, ZD1839 (Iressa) is an EGFR tyrosine kinase inhibitor currently in phase 2 clinical evaluation for treating advanced breast cancer (298,299). Perhaps drugs could be created that act as selective coregulator modulators to work in concordance with SERMs to alter coactivator and corepressor concentrations in selected tissues to increase the agonist (bone and vasculature) or antagonist (breast and uterus) effects of drugs such as tamoxifen and raloxifene (300).

Current advances in the treatment and prevention of breast cancer have been built on identifying a target, the ER, and applying existing knowledge to create drugs to specifically block ER signaling. The extensive study of tamoxifen over the past 30 yr has produced a road map of experience for the next generation of basic and clinical inves-

tigators. It is clear that the future holds exciting prospects for discovery of a wide range of new agents to target different risk factors for osteoporosis, coronary diseases, and uterine and breast cancer.

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# 11

## Cyclin-Dependent Kinases and Their Regulators as Potential Targets for Anticancer Therapeutics

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### Introduction

A number of complex changes take place between the time a cell is formed and the time it divides into two daughter cells. This process is known as the cell cycle. The morphologic changes associated with particular stages of the cell cycle are well known; however, a detailed understanding of the regulatory mechanisms controlling cell-cycle progression has only recently been elucidated. Understanding the biochemical and genetic mechanisms that control these cellular changes is fundamental to cell biology because it influences processes such as cell transformation, cell differentiation, and cell growth. A greater knowledge of the molecular mechanisms underlying the transformation of mammalian cells may allow the design of inhibitors of the specific biochemical processes responsible for abnormal cell proliferation or cancer.

The core of the cell-cycle machinery has been preserved through evolution. As organisms evolved, cells adapted to respond to a larger and more complex number of stimuli that dictate their proliferative activity. This evolution has usually been achieved by the addition of more layers of control over the same basic cell-cycle circuitry. The use of yeast and invertebrate systems has provided invaluable information and experimental tools to aid our understanding of cell-cycle regulation (1,2). This chapter deals with the group of proteins directly involved in the regulation of mammalian cell division. These regulatory proteins belong to a unique family of kinases named cyclin-dependent kinases (CDKs). The identification of CDKs has led to a number of other related and important discoveries of the molecular mechanisms involved in the regulation of cell-cycle progression. A number of protooncogenes (*cyclin D1*, *CDC25*, *CDK4*) and tumor suppressor genes (TSGs) (*pRb*, *p53*, *p16*) have been identified in the context of cell-cycle regulation. Together, these discoveries have enhanced our overall understanding of cell transformation and tumor biology.

This chapter reviews CDKs, and their role in cell transformation and cancer. The potential applications of CDKs in rational drug design for the development of new antiproliferative agents in oncology is discussed. Inhibition of CDKs represents one of the first mechanistic approaches to the development of therapies for cancer.

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Soon after the publication of the first edition of this book, three investigators in the field of cell-cycle control were awarded the Nobel Prize for Physiology and Medicine: Leland H. Hartwell, R. Timothy Hunt, and Paul M. Nurse received this award for the confluence of two different approaches to learning about the molecular machinery regulating the cell cycle.

While Hartwell was screening for temperature-sensitive mutants in the yeast *Saccharomyces cerevisiae* in the 1960s and 1970s, he identified a large series of genes that, in mutant form, arrested mitosis. This led him and Ted Weinert to hypothesize that “checkpoints” regulate the sequence of events in mitosis (3). Even as Hartwell was uncovering these genes, Nurse was identifying in a different yeast, *Schizosaccharomyces pombe*, CDC2, a protein kinase that is a key component of the maturation factors that drive cell division (4). He took a human cDNA library and eventually cloned a human CDC2, observing the striking structural homology of these key regulatory molecules, indicating a high degree of evolutionary conservation in different species.

Working at the Woods Hole Marine Biological Laboratory in the early 1980s, Hunt produced a crucial piece of the cell-cycle puzzle. Using sea urchin oocytes and frogs, Hunt identified proteins whose concentrations increased and decreased throughout the cell cycle (5). He called them cyclins, and they were later found to be necessary in activating the always present kinases, such as CDC2. Together, a cyclin and a CDK push the cells along the cell-cycle regulatory checkpoints.

Other researchers did important early work in the field. In the early 1970s, Yoshio Masui and L. Dennis Smith (6,7) transferred the cytoplasm from activated frog oocytes to naive oocytes arrested in meiosis and identified a substance they called MPF (maturation promotion factor), which 20 yr later proved to be CDK/cyclin heterodimer.

In 2002, the Nobel Prize was awarded for seminal discoveries concerning another key issue of biologic relevance: the genetic regulation of organ development and programmed cell death, or apoptosis, to Sydney Brenner, John Sulston, and Robert Horvitz. Together they discovered that specific genes control the cell death program in the nematode worm *Caenorhabditis elegans*. This 1-mm-long worm has a short generation time and is transparent, which makes it possible to follow its cell division under the microscope. In this organism, all cell divisions and differentiations are invariant, i.e., identical from individual to individual. This made it possible to construct a precise cell lineage for all cell divisions. During development, 1090 cells are generated, but precisely 131 of these cells are eliminated by programmed cell death, producing an adult nematode composed of 959 somatic cells. Most of the multiple genes controlling apoptosis have human equivalents. Work from the Human Genome Project suggests that there are between 30,000 and 40,000 genes in the human genome, and 18,000 in the nematode worm. Both the cell-cycle control genes and the apoptosis genes are crucial for carcinogenesis and are targets for cancer therapy. Optimal curative cancer therapies should include combinations of drugs that not only cause cell-cycle arrest in tumor cells, but also lead to their apoptosis.

Growth and differentiation must be tightly controlled in any organism. Research suggests that fractal networks for transporting materials essential to life, such as, nutrients, water, and oxygen, may be a prime mover in determining shape and form in nature. A series of key articles by West and colleagues (8,9) has offered three general principles for allometric scaling laws, which are major determinants of most anatomical and physiologic variables of organisms as they increase in body size.

Thus, in this early part of the twenty-first century, we are beginning to understand growth and differentiation both from the point of view of their general laws and relatively complex mathematical models, and from the point of view of molecular and reductionistic mechanisms.

Probably the better known growth factor family involved in setting up the basic body plan during early embryogenesis in mammals, frogs, and flies is the transforming growth factor- $\beta$  (TGF- $\beta$ ) family. These cytokines play a major role in the control of the formation of cartilage, bone, and sexual organs; suppress epithelial cell growth; foster wound repair; and regulate complex immune and endocrine functions.

Research by Massagué and colleagues (10–13) has led to the partial elucidation of some of the intervening molecules and steps, from the cell-surface receptors to the nucleus. These steps involve at least two related transmembrane protein kinases. The first, known as R-II, acts as a primary receptor that binds TGF- $\beta$  directly from the medium or from an auxiliary ligand-presenting protein known as betaglycan. Once bound to R-II, TGF- $\beta$  is recognized by the second transmembrane kinase, R-I, which becomes phosphorylated and activated by R-II. This activation leads to propagation of the signal from the cell membrane to the nucleus. Through intervening steps yet to be completely defined, the pathway initiated by the TGF- $\beta$  receptor complex leads to inhibition of CDK by the action of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), also known as p27, and the related p21 and p57 molecules.

p53 can induce the expression of p21, to arrest the cell cycle and allow for sufficient time for DNA repair after some types of DNA damage (14,15). The p53 gene is mutated in as many as 50% all human tumors, and by now 15,000 different mutant forms of this regulatory protein have been described (16).

Even if p53 is not mutated, overexpression of the p53 antagonist HDM2 (the human form of the murine double minute 2 protein), or deletion or underexpression (by hypermethylation of the promoter) of the HDM2 antagonist p19<sup>ARF</sup> (belonging to the INK4 family of CKIs) can equally lead to increased degradation of p53, thereby avoiding the probability of cell-cycle arrest (17).

Thus, a diverse array of biochemical changes all converge on a common regulatory pathway and, in the presence of the correct genetic and molecular alterations, lead to or reinforce the malignant phenotype of cancer cells.

## CDKs and Their Role in Cell-Cycle Progression

Each cell division cycle consists of two major periods of activity (Fig. 1). The first period of activity, or S-phase, involves DNA replication in which the cell duplicates its genetic material. The second period, or M-phase, occurs when the sister chromatids separate and nuclear division takes place, generating two nuclei with identical and complete sets of genetic material. In mammalian cells, the M-phase is also kinetically linked to the process of cytoplasmic division or cytokinesis. The S-phase and M-phase are separated by two Gap phases (G1 and G2). The complete cell cycle proceeds through G1- to S-phase to G2-, and finally to M-phase. The transition through these phases of the cell cycle is regulated by the CDKs. Other important events, including checkpoint controls (checkpoint controls ensure fidelity in the completion of the critical processes in the preceding phases), occur during the G1 and G2 phases. The G1 and G2 processes ensure proper cell growth and accumulation of critical cellular components required for the advancement of the cell to the next phase. The restriction point or R point, located in late G1, is critical to cell-cycle progression. R defines the point

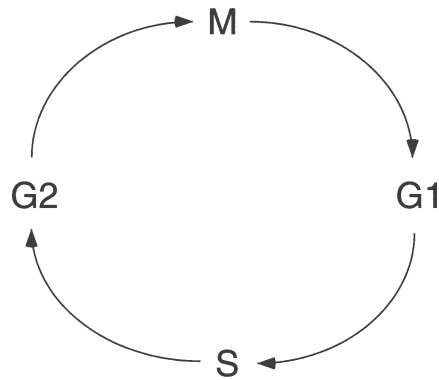


Fig. 1. Cell-cycle phases. See text for description of G1, S, G2, and M.

beyond which cell-cycle progression becomes independent from external growth factors and thereby committed to that round of cell division (18).

Protein phosphorylation is a reversible modification that has been implicated in cellular regulation of protein structure and activity; this occurs by phosphorylation of specific residues through adenosine triphosphate (ATP) hydrolysis and phosphoester bond formation. The CDKs are heteromultimeric kinases (19,20) that phosphorylate specific Ser/Thr residues adjacent to Pro in their protein substrates. Other basic residues surround these two positions. The primary sequence is not the only determinant for CDK substrate utilization (21,22). For example, CDK4/cyclin D1 and CDK2 cyclin E both phosphorylate retinoblastoma protein (pRb) but show preference for different positions on the substrate (23). The activities of the CDK complexes can themselves be modulated by phosphorylation.

In their simplest form, CDKs are dimeric complexes composed of a catalytic subunit, which contains the ATP-binding pocket, and a regulatory subunit. The composition and stoichiometry of the specific complexes vary depending on the individual components, stage of the cell cycle, cell type, and transformation state of the cell (24). To be catalytically competent, the catalytic subunit (CDK1–9) and a regulatory subunit (cyclin A–H and cyclin T) must combine and be in the correct phosphorylation state. Figure 2A shows the combinations of the catalytic and regulatory subunits that furnish functionally discrete CDK complexes. Alternative spliced forms (i.e., cyclin E) and additional subtypes (i.e., cyclins D1–3 or cyclins A1–2) enlarge the number of cyclins and the complexity of the functional CDK/cyclin combinations. The activity of most of the CDK complexes can be associated with specific points during cell-cycle progression (20) (Fig. 3); however, some family members are associated with processes that do not involve proliferation. CDK 5, for example, is involved in neuronal processes and not proliferation (25), and other CDK complexes are involved in transcriptional control (26).

Whereas the levels of the catalytic CDK subunits remain relatively constant throughout the cell cycle, the amounts of the regulatory cyclin subunits change. These changes mean that specific cyclins will be present only at certain times during the cell-division cycle. Consequently, the cyclins regulate CDK complex formation and activity and also confer substrate specificity and cellular compartmentalization. The cyclins contain an amino acid motif (approx 100 amino acids) known as the cyclin box; this cyclin

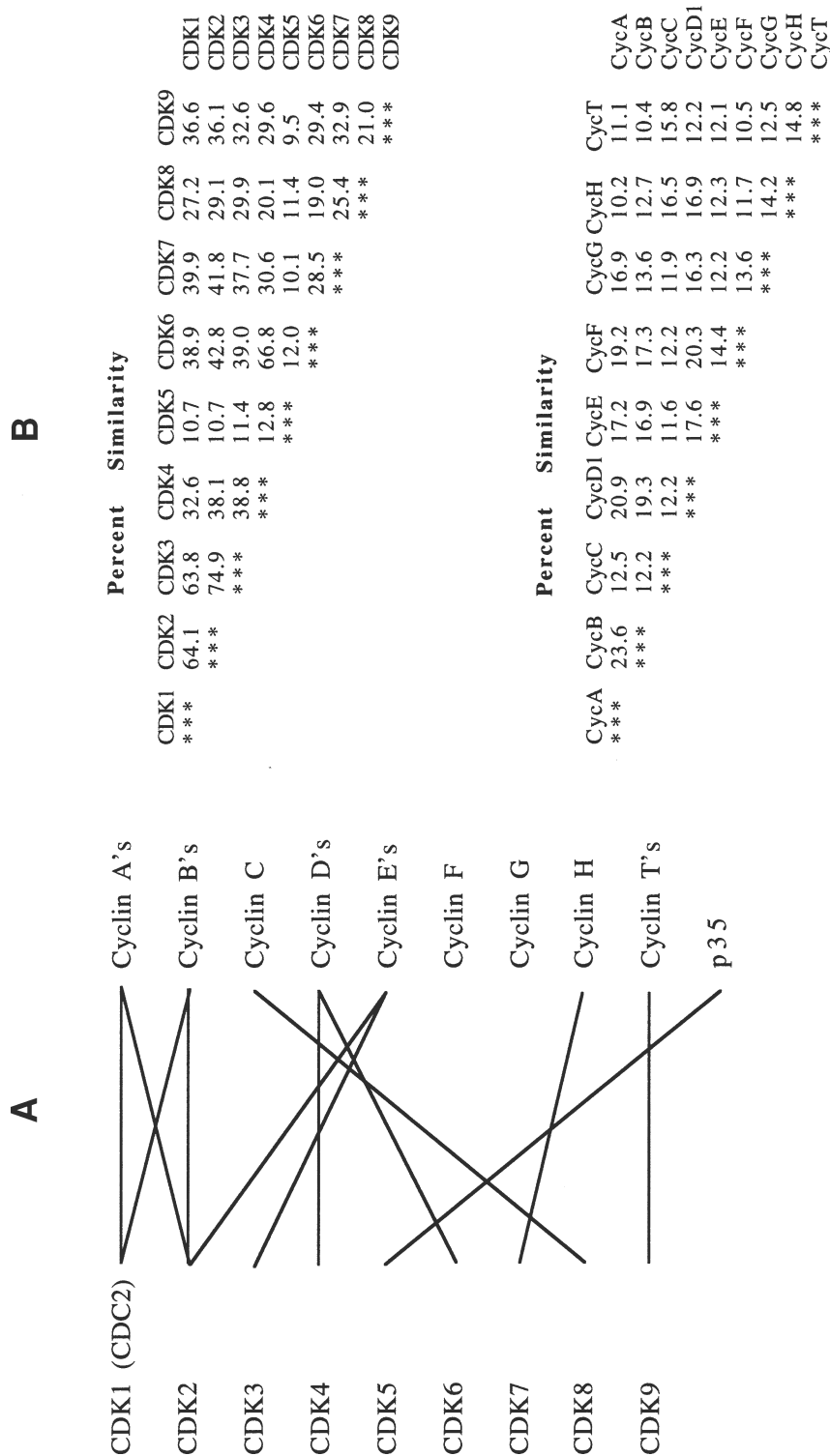


Fig. 2. CDKs and cyclins. (A) CDK and cyclin combinations found in vivo; (B) similarity among CDK family members.

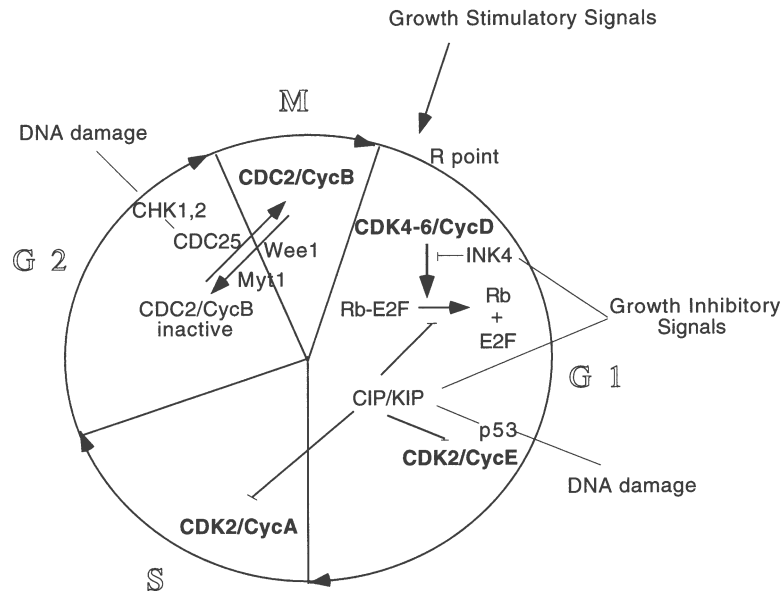


Fig. 3. CDK control of cell-cycle regulation.

box is also found in pRb and TFIIB. This motif is a critical element at the CDK–cyclin interface and defines a 5  $\alpha$ -helix bundle that is repeated twice in the cyclin subunit (27,28). The cyclins show high structural similarity but their overall level of identity is very low (Fig. 2B). Although the smallest kinases known, the CDK catalytic subunits contain all the usual kinase subdomains plus a characteristic signature sequence (variations of the “PSTAIRE” sequence) that define a key element for interaction with the cyclins. Experiments indicate that some of the CDK/cyclin complexes may show some level of redundancy. For example, despite the decreased size and defects in mammary epithelial and retina cells displayed by the cyclin D1 knockout mice, cyclin D1 deletion does not result in embryonic lethality or any major postembryonic collapse (29).

Figure 3 shows the distribution and activities of a number of the CDK complexes involved in cell-cycle regulation. CDKs integrate signals from the cell and its environment to either initiate or inhibit cell-cycle progression. Granulocyte-macrophage colony-stimulating factor (GM-CSF), for example, induces increased cyclin D1 synthesis in a fashion that parallels the stimulatory activity of this growth factor (30). Other signals such as the mitogen antagonists cyclic adenosine monophosphate (cAMP) and TGF- $\beta$  have a negative effect on CDK and cell-cycle progression (31,32). DNA-damaging agents and differentiation factors also induce cell-cycle arrest through CDK modulation (33,34).

A number of other proteins are known to interact with these subunits: the proliferating cell nuclear antigen (PCNA), CKI proteins (CIP-KIP and INK4 family members), the members of the retinoblastoma (Rb) family (p107/p130/p300), transcription factors, and various transforming viral proteins (20,24). The significance of each of these interactions is not completely understood.

Checkpoint controls monitor the state of completion and fidelity of the critical molecular and macromolecular processes preceding critical commitment points in the



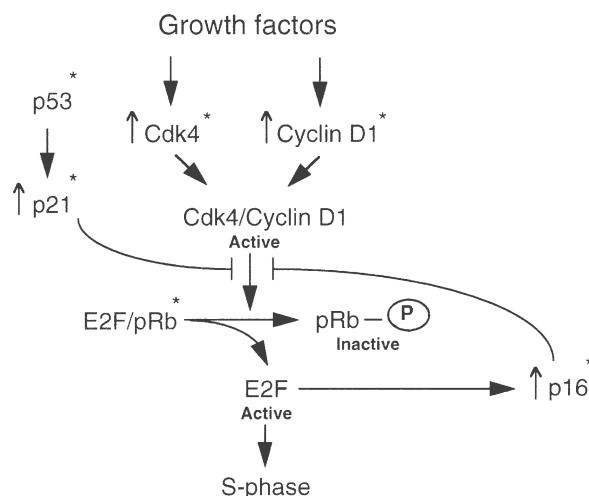


Fig. 4. The CDK4/cyclin D/pRb/p16 pathway. Asterisks indicate that alterations in the respective genes have been associated with cellular transformation and tumorigenesis.

cell-division cycle. CDKs play a critical role in the execution of the cell-cycle checkpoints. To date, two cell-cycle checkpoints have been characterized. The first precedes DNA synthesis (S-phase) and ensures replication of undamaged DNA (33,34). The second immediately precedes mitosis (G2/M-phase) and is affected by DNA damage, incomplete DNA replication, or spindle microtubule polymerization (35). CDK1 (CDC2) has been identified as the final mediator of the G2/M-phase checkpoint (36). Deregulation of checkpoint elements has been reported to be associated with virally induced oncogenesis (37).

Execution of the restriction point and activation of CDK (CDK4 and CDK6) are closely associated. CDK4/CDK6 activity is required for entry into G1 and subsequent cell-cycle progression (38) (Fig. 3). CDK4–cyclin D phosphorylates the *Rb* TSG that leads to its dissociation from the E2F transcription group of proteins. This dissociation allows the E2F–DP complexes to initiate the transcription of a large number of genes required for DNA synthesis. Whereas pRb contains 16 potential CDK phosphorylation sites, CDK4/cyclin D preferentially phosphorylates S-795 (23). Although elimination of the CDK4/cyclin D complex arrests cell-cycle progression in *Rb* wild-type cells, it has no effect on cells lacking functional pRb (23). These results support the hypothesis that pRb is the only physiologically relevant substrate of CDK4. Other elements involved in the pRb pathway are the CKIs p16 (and related members) and p21 (and related members), as well as tyrosine kinase(s) involved in negative regulation of CDK4 (39). Multiple oncogenic alterations have been associated with the *Rb* pathway, including activation of several oncogenes and inactivation of diverse tumor suppressors (Fig. 4), highlighting its central role in the regulation of cell proliferation and transformation.

CDK2/cyclin E is required for G1 progression; pRb is again a key substrate. As in the case of cyclin D, ectopic expression of cyclin E shortens G1. It appears that pRb must be phosphorylated by CDK4/cyclin D before pRb phosphorylation by CDK2/cyclin E (40). Moreover, elimination of the pRb function does not eliminate the need

for CDK2/cyclin E for cell-cycle progression (41,42). This finding suggests that substrate(s) other than pRb may mediate the cell-cycle regulatory role of CDK2/cyclin E. In mammalian cells, CDK2/cyclin A has been associated with control of DNA synthesis. Cyclin A localization to the nucleus is associated with the start of S-phase. Microinjection of antibodies against cyclin A, as well as expression of antisense constructs, inhibit DNA synthesis (43). CDK1/cyclin B activity is responsible for induction of mitosis in all eukaryotic cells. This activity, MPF, constitutes the cellular histone H1 kinase (44–46). MPF is a dominant activity able to induce mitotic events in cells regardless of their position in the cell cycle (47). Not only is CDK1/cyclin B activation absolutely required for entry into mitosis, but its elimination or degradation is necessary for cells to exit mitosis. Cyclin B mutants devoid of the destruction box sequence necessary for degradation cannot eliminate cyclin B and hence induce a mitotic block (48). Cyclin B degradation occurs through ubiquitin-mediated degradation by the anaphase-promoting complex (49). Substrates other than histone H1 for CDK1/cyclin B have been identified and consist of a range of structural proteins and enzymes that are involved in the morphologic changes that occur during mitosis, including lamins, vimentin, caldesmon, microtubule-associated proteins, CDC25 (as part of a feedback loop control), cABL, and CKII (50).

### Regulation of CDK Activity

The molecular and biochemical basis of CDK regulation is best understood through familiarity of the three-dimensional (3D) structures of these molecules and their regulators. Atomic models, obtained by crystallographic and nuclear magnetic resonance analysis of CDK2, CDK6, cyclin A, cyclin H, and some of their inhibitors (p27, p19, p18, and p16) in various monomeric and bound forms, have provided valuable information regarding the structural implications of their primary sequences, as well as the role of critical posttranslational modifications and atomic and structural effects of their interactions.

The catalytic CDK subunits form a bilobular structure typical of known protein kinases (Fig. 5) (28,51–53). The smaller lobe of the catalytic subunit contains approx the first 100 residues of the protein and comprises a five-stranded  $\beta$ -sheet and a unique  $\alpha$ -helix. The  $\alpha$ -helix contains the signature CDK PSTAIRE motif and is responsible for interaction with the regulatory cyclin subunit (red domain in Fig. 5) (28). The larger lobe, defined by approx 200 residues of the C-terminal, mainly comprises  $\alpha$ -helices and is predicted to contain the peptide-binding site. The ATP-binding site lies in the cleft between the two lobes of the catalytic subunit (51,54). The small lobe contains the highly conserved Gly loop that provides the backbone amides that hydrogen bond to the  $\beta$ - and  $\gamma$ -phosphate of ATP and the highly conserved Lys residue (E51 in CDK2) involved in ion pairing with the  $\alpha$  and  $\beta$  phosphates of ATP. Key Thr and Tyr residues (T-14 and Y-15, respectively, in CDK2) involved in the negative regulation of CDK activity lie in the Gly-rich region. The large lobe encodes the critical aspartic acid (D146 in CDK2) that establishes salt bridges with E51 and defines the correct configuration of the ATP-binding pocket. Another important motif of the CDK is the T loop, which contains the CDK-activating kinase (CAK) phosphorylation site (T-160 in CDK2) (orange motif in Fig. 5 and Fig. 6) required for kinase activation.

The monomeric catalytic CDK subunit is catalytically incompetent. Binding the regulatory subunit induces a number of changes that allow proper ATP binding and catalysis to occur (28,51). The signature PSTAIRE loop rearranges to bring the E51 in

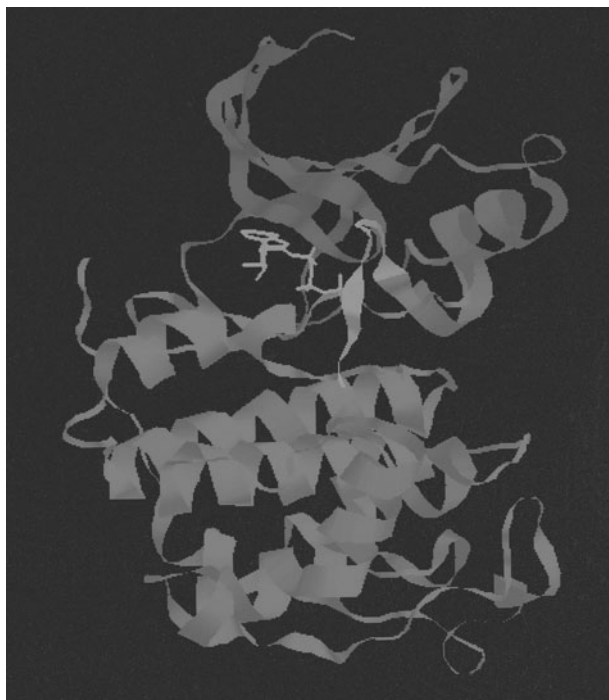


Fig. 5. Crystal structure of monomeric CDK2. Purple, small lobe; magenta, large lobe; red, PSTAIRE helix; orange, T loop; green, ATP. (Color illustration in insert following page 362.)



Fig. 6. Crystal structure of CDK2 bound to cyclin A. Magenta, CDK2; blue, cyclin A; red, PSTAIRE helix; orange, T loop; green, ATP. (Color illustration in insert following page 362.)

proximity to the ATP-binding site. This stabilizes the position of the active site Lys to allow proper orientation of the ATP. In a second conformational change, the T loop moves away from the catalytic cleft (compare the position of the T loop in Figs. 5 and 6). CAK phosphorylation of the T-160 residue further stabilizes the T loop by eliminating the steric hindrance that the T loop places on the catalytic site and allowing access to the substrate (55).

### Posttranslational Modifications of CDK

As with other protein kinases, the activity of CDK is regulated by phosphorylation/dephosphorylation events. To be catalytically competent, the newly formed CDK–cyclin complex must be correctly phosphorylated at position T-160 in the T loop. The kinase responsible for T-160 phosphorylation is CAK (56). CAK (CDK7/cyclin H), while a member of the CDK family (Fig. 2A), does not show cell-cycle-dependent activity. CAK has been associated with the transcriptional machinery as part of the TFIIH complex involved in the phosphorylation of the C-terminal repeat of the RNA polymerase II (57). In budding yeast, the dimeric CAK homolog (CDK7/cyclin H) has been associated only with transcriptional control, while a novel monomeric kinase, CAK1 (unrelated to CAK), has been associated with positive regulation of its substrate, CDC28 (58).

Phosphorylation can have a negative role in the regulation of CDK. Phosphorylation of residues T-14 and Y-15 of CDK2 (and equivalent residue on other CDKs, when applicable) has a negative effect on CDK activity (59). The T-14 and Y-15 residues are located in the glycine-rich loop of the kinase that forms the roof of the ATP-binding site and is involved in critical interactions with the ATP. At the G2- to M-phase transition, the dephosphorylation of T-14 and Y-15 residues of CDK1/cyclin B complex is the limiting step for activation of this kinase and for induction of mitosis (60). Phosphorylation of the T-14 and Y-15 positions is performed by Myt-1 (a dual specific kinase) or Wee-1 (a tyrosine kinase) (61,62). The inhibitory effect of T-14 and Y-15 phosphorylation can be reversed by the CDC25 family of dual specific phosphatases (60). The CDC25 family has three members: CDC25A, B, and C. The oncogenic potential of CDC25A and B has been described (63), but CDC25C is the best understood of these three phosphatases. CDC25C has been associated with dephosphorylation and activation of CDK1/cyclin B at mitosis. The activity of the CDC25C phosphatase is itself regulated by two mechanisms (Fig. 7). As part of a positive feedback loop, CDC25C becomes phosphorylated by its substrate, the CDK1/cyclin B kinase (64,65). In addition, CDC25C is regulated by its interaction with the 14-3-3 proteins (66,67). Interaction of the phosphatase with the 14-3-3 proteins requires phosphorylation of the former at position 416 by Chk 1, Chk 2, and c-TAK1 (68–70). This interaction results in downregulation of CDC25 activity and/or in its altered cellular localization (66,67).

### Cellular Inhibitors

A large number of proteins have been identified that bind to and inhibit the CDK–cyclin complexes. These proteins are generically referred to as CKIs (71). CKIs can be divided into two groups: the INK4 and CIP–KIP families of inhibitors (Fig. 8). The INK4 (inhibitors of CDK4 or 6 kinases) family members—p15, p16, p18, and p19—are structurally highly conserved. They bind and inhibit CDK4- and CDK6-associated kinases specifically (72). Although the INK4 members behave identically in vitro, their

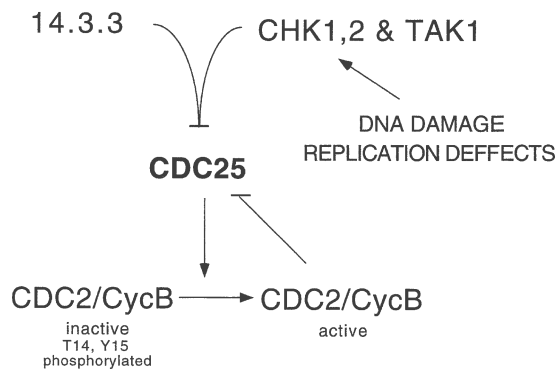


Fig. 7. Regulation of CDC25C.










CDK Inhibitor	Modulator	Targets
<u>INK4 Family</u>		
p16 	RAS, Senescence	CDK4,6
p15 	TGF-β	" "
p18, p19, p20 	?	" "
<u>Cip / Kip Family</u>		
p21 	p53 MyoD	CDK2,4,6,1
p27 	Cell contact cAMP Rapamycin TGF- β	" "
p57 	?	" "
<div> Ankyrin Repeat</div> <div> CDK Inhibitory Domain</div> <div> PCNA Binding Site</div>		

Fig. 8. Cellular CKIs: INK4 and CIP/KIP families.

cellular expression patterns differ and they respond to different signals. Upregulation of p16 has been observed in cells by activated Ras and during senescence (73). Amounts of p15 change the response of cells to TGF-β or other antimitogenic agents (74). The expression of p18 and p19 is regulated in a cell-cycle-dependent manner with maximum expression at S-phase (75).

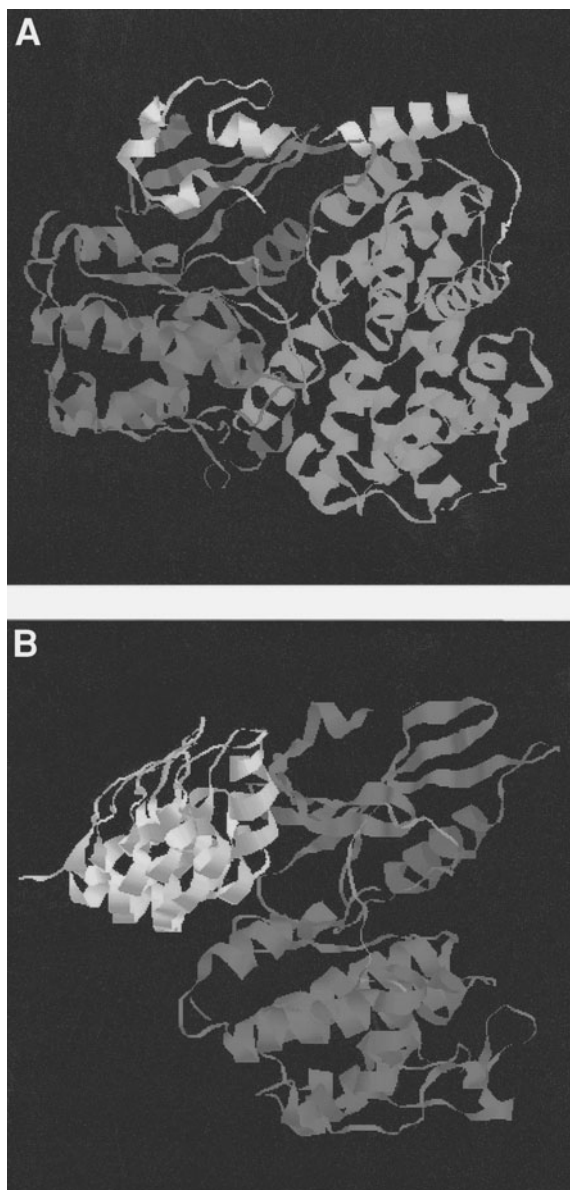


Fig. 9. (A) Crystal structure of CDK2–cyclin A bound to p27. Magenta, CDK2; blue, cyclin A; white, p27 N-terminal domain. (B) Crystal structure of CDK6 bound to p19. Magenta, CDK6; white, p19.(Color illustration in insert following page 362.)

Analysis of the CDK6–p19 structure (52,53) (Fig. 9) suggests that p19 exerts its inhibitory activity by changing the structure of the CDK6 molecule. The INK4a locus encodes two proteins, p16 and p19<sup>ARF</sup>, which regulate two important tumor suppressor pathways involved in human cancers. p16 regulates the pRb pathway (through the modulation of cyclin D–dependent kinases) (72). p19<sup>ARF</sup>, which is generated by an alternative reading frame (ARF) within this locus, induces p53 stabilization (76).

Mice that were originally engineered to eliminate the p16 expression, but that also eliminate p19<sup>ARF</sup>, develop sporadic tumors at an early stage and show hypersensitivity to chemical carcinogenesis (77). Mice lacking the function of only p19<sup>ARF</sup> also develop sporadic tumors, validating it as a bona fide tumor suppressor (78).

Members of the CIP–KIP family inhibit all the CDK complexes and show little structural similarity. CIP–KIP members (Fig. 8) have an important role in the control of cell proliferation in response to antimitogenic agents. For example, p21 and/or p27 can regulate cell-cycle arrest induced by differentiation factors (in a p53-independent fashion) (79), DNA-damaging agents (in a p53-dependent fashion) (33,34), and antimitogenic conditions such as cell-to-cell contact and loss of cell anchorage (79). Modulation of p27 levels regulates cell proliferation (80). Correlation of low levels of p27 expression in tumor samples with poor patient survival indicates that p27 regulation has an impact in normal development and in aberrant cell growth. Several reports indicate that the cellular levels of p27 are regulated at the transcriptional, translational, and post-translational levels (71). Phosphorylation and ubiquitin-mediated degradation (81) have been reported to be involved in regulating p27's stability.

The structure of CDK2/cyclin A in the presence of the inhibitory domain of p27 has been reported (82). This structure shows that the N-terminal region of p27 interacts with the small lobe of CDK2, thereby altering the conformation of this region and the ATP-binding site. One of the key interactions outside the ATP-binding pocket involves the N-terminal coil of p27 and a highly conserved shallow groove on the cyclin molecule. This interaction is defined by the RRLFG motif of p27 that is also present in other proteins known to interact with CDK/cyclin complexes (83). Structural information derived from a CDK2/cyclin A/p27 complex corroborates genetic and biochemical data indicating that motifs of p21 and p27 interact with both the CDK and cyclin subunits (84). A role for CIP members in modulation of complex formation of cyclin D–dependent kinases has been proposed based on this observation. Finally, reshuttling of p27, from cyclin D complexes to cyclin E complexes, as cells are subjected to mitotic stimuli has also been reported (85).

### ***Some Other Mechanisms of Regulation***

CDK activity is also regulated by mechanisms other than posttranslational modifications and interaction with cellular inhibitors. As discussed earlier, synthesis and degradation of the regulatory cyclin subunit is another important factor. Ubiquitin-mediated protein degradation has a central role in regulating cyclin levels. This was originally shown for cyclin B and later observed with cyclins E and D (86–88). Likewise, ubiquitination has been linked to p27 degradation. Phosphorylation at specific positions is believed to be the trigger for this degradative pathway. Another level of regulation takes place at the transcriptional level. Upregulation of cyclin D transcription in response to growth factors and induction of p21 and p15 in response to antimitogenic signaling are examples. Cellular localization of the enzymes with respect to the relevant substrate(s) is another level of regulation. For example, cyclin B1 is found in the cytoplasm until the initiation of mitosis, when it moves to the nucleus. A specific sequence, the cytoplasmic retention signal, is responsible for sequestering cyclin B1 in the cytoplasm (89).

Another level of regulation is represented by modulation of the CDK/cyclin complex assembly. CDK4/cyclin D complex formation requires mitogenic signals. The necessary serum stimulation can be mimicked by ectopic expression of MEK1 and is likely to involve modulation of interaction of CDK4 with molecular chaperons such

as CDC37 (90,91). Also, a phosphatase candidate for the T-loop dephosphorylation has been cloned (92) and has been suggested to preferentially use uncomplexed, T-loop phosphorylated CDKs as substrates (93). However, no clear indication of its regulatory nature with respect to cell-cycle control has been described. Finally, it is important to mention that regulation of the interaction between CDK complexes and a subset of cellular and viral proteins (including CDK substrates) is based on the use of a common motif present in a number of CDK-interacting proteins, including p21 family members, E2F, CDC25, and p107 (93). This is the "RRLFG" motif, in the context of the p27 and CDK2/cyclin A interaction. As discussed, differential CDK inhibition has been shown with peptides containing this sequence (94). Cellular effects of such peptides are described later.

### ***Oncogenic Alterations of Cell-Cycle Regulators***

Normal cells transition from G1- to S-phase in response to extracellular signals such as growth factors, hormones, or cytokines, or after contacts with other cells and the extracellular matrix (ECM). In the absence of these extracellular signals, the cells withdraw to quiescence (G0) from which they may either reenter into the cell-division cycle or differentiate. Again, both of these decisions are made in response to specific extracellular signals. This mechanism ensures the correct balance of growth of the various tissues. It has long been recognized that the breakdown in these growth control processes is a hallmark of cancer (16,94).

In contrast to G1- to S-phase, the G2- to M-phase transition is regulated mainly by intracellular signals (such as the completion of DNA synthesis). The role of the G2- to M-phase checkpoint is to prevent mitosis when the DNA is damaged and not repaired. Alterations that abrogate the G2- to M-phase checkpoint control allow cells with damaged genomes to undergo mitosis and result in the transmission of mutated genomes. From these altered genomes, new mutations may arise that contribute to the selection of cancer cells. This genomic instability is another hallmark of cancer (16,94).

Since the discovery of the various mammalian cell-cycle regulators, many reports have examined the expression of various cell-cycle regulators in human tumors. The inherent problem of this approach is that it can only suggest a correlation. It is difficult to differentiate whether cells proliferate uncontrollably because they contain abnormal amounts of the specific cell-cycle regulatory protein, or whether this protein is present in abnormal quantities only because the cells have divided uncontrollably. This section focuses on some molecular alterations of the cell-cycle regulators in which the genetic alteration in the gene itself is detected, or in which the abnormal RNA or protein levels may have diagnostic/prognostic value for the treatment of the tumor. Other, more recent molecular correlations between particular cyclin levels and certain cancers are mentioned below.

### ***Alterations of Cyclin D1 in Human Tumors***

In the past decade, molecular analysis of tumors has revealed a number of different mechanisms that lead to the deregulated expression of *cyclin D1* (*CCND1*) gene. Indeed, the number of tumors that show alteration in the cyclin D pathway (either in the cyclins themselves or in their upstream regulators or downstream targets; Fig. 4) consistently implicate *CCND1* in the development of a variety of human tumors. These data suggest that the deregulation of this pathway is extremely important for the development of human cancers and holds promise for therapeutic intervention.



### *Chromosomal Translocations Affecting CCND1*

*CCND1* maps to 11q13, a region that is altered in a variety of proliferative disorders (95). In a number of hematologic malignancies, reciprocal chromosomal translocation is a common feature. These translocations result in either the deregulated expression of a gene lying close to the breakpoint or the fusion of the coding information from the two chromosomal partners (96). One of the characteristic translocations in a group of B-cell neoplasms (now collectively called mantle cell lymphoma, [MCL]) is the t(11;14) translocation in which the *BCL-1* locus on chromosome 14 becomes juxtaposed with *CCND1* on chromosome 11. As a result, most tumors with the t(11;14) translocations show increased expression of the cyclin D1 RNA, protein, or both, arguing that the primary target gene activated by the translocation is *CCND1* (97). It appears that most MCLs, which account for approx 5% of all non-Hodgkin's lymphomas (NHLs), show cytogenetic or molecular evidence for the t(11;14) translocation (98,99). However, a significant number of MCLs without the apparent translocations expresses increased amounts of *CCND1* (100,101). The mechanism(s) responsible for the overexpression in the latter situation remain unknown. Interestingly, none of the translocations that have been examined so far affected the coding region of *CCND1*, suggesting that the normal gene product contributes to tumorigenesis.

### *Other Chromosomal Rearrangements Affecting CCND1*

Another type of chromosomal rearrangement that also activates *CCND1* expression is an inversion of part of chromosome 11 (inv[11][p15;q13]) that places the *CCND1* locus at the band q13 adjacent to the parathyroid hormone gene (*PTH*) at band p15 (102). This rearrangement has been reported in only three cases of benign parathyroid adenomas, but the impact of this rearrangement was enormous, because it led to the discovery of *CCND1* as the candidate *PRAD1* oncogene (103). In each case, the rearrangement resulted in a dramatic increase in the expression of cyclin D1 RNA, thereby contributing to the formation of the adenoma.

### *Gene Amplifications Affecting CCND1*

The most frequent chromosomal abnormality that affects *CCND1* is the amplification of the 11q13 region that had been observed in a significant portion of breast and squamous cell carcinomas. The amplification of this region suggests that a potential oncogene(s) lies in the area providing a selection pressure for the maintenance of the amplicon. Several lines of evidence suggest that this candidate oncogene is *CCND1*. First, as the consequence of the amplification, *CCND1* is expressed at higher levels (104–106). Second, in most 11q13 amplifications, *CCND1* lies at the center of the amplification unit and is amplified (107,108). The average amplification frequency of *CCND1* in primary breast tumors is 13–24% and the cyclin D1 protein is overexpressed in approx 50% of the tumors (109–113).

High-frequency *CCND1* amplifications and *CCND1* overexpression are also observed with squamous cell carcinomas of the head, neck, oral cavity, larynx, and esophagus (114–117). *CCND1* overexpression has been observed in approx 50% of these cases, and *CCND1* amplification has been detected in 23–40% of the cases. The *CCND1* locus is amplified in 32% of non-small cell lung carcinomas (NSCLC) and 44% of the tumors overexpress *CCND1* (118). In pancreatic carcinomas, the data are more controversial. One study found *CCND1* amplification in 25% of the samples and detected *CCND1* overexpression in 68%. This study also found a correlation between the nuclear

overexpression of *cyclin D1* and the poor prognosis of the tumor (119). In a similar study, no *CCND1* amplification was detected (120).

### ***CDK4 Amplification in Human Sarcomas and Gliomas***

The *CDK4* gene encoding the catalytic partner of cyclin D1 is located on chromosome 12q13 (121) and lies in a region that is frequently amplified in human sarcomas and gliomas (122,123). Several genes, such as *WNT1*, *MDM2*, *GADD153*, *GLI*, *OS4*, *GAS16*, *GAS27*, *GAS41*, *GAS56*, *GAS64*, *GAS89*, and *SAS*, are implicated in the development of subsets of cancers and lie in this region (124). However, several studies argue that *CDK4* amplification is the key event (125–127). It has been demonstrated that *CDK4* is overexpressed as the result of amplification (127,128). *CDK4* amplification and overexpression show a reciprocal correlation with the deletion of its inhibitor p16 and its regulatory subunit cyclin D1 (129–133). The reported amplification frequency of *CDK4* in sarcomas is 8–36% (128,134) while in gliomas and astrocytomas this frequency is approx 10% (127,135).

### ***Mutations in p16-Binding Domain of CDK4 in Familial Melanomas***

Analysis of familial melanoma patients has revealed an interesting mutation in the coding region of *CDK4* that also illustrates the intimate relationship between *CDK4* and *p16* and the spectacular diversity of molecular alterations that affect the cyclin D1/CDK4/p16/Rb pathway in human tumors. In approx 5% of the familial melanomas, the *R24* in *CDK4* is mutated to *C24* (136–138); the X-ray structure shows that the *R24* is buried in the protein-protein interface. This mutation, which functionally maps to the p16-binding domain of *CDK4*, disrupts the interaction between *CDK4* and the p16 family of inhibitors, leading to the deregulation of the D1/CDK4/p16/Rb pathway (16).

### ***Deletion and Point Mutations That Inactivate p16<sup>Ink4a</sup>/MTS1/CDKN2A***

Two independent lines of research led to the discovery of p16 as an inhibitor of the CDK4/cyclin D kinase (72) and also implicated it as a candidate tumor suppressor located at the chromosomal position 9p21 (139,140). This chromosomal region is frequently deleted in many human tumors and is linked to hereditary susceptibility to melanoma (141,142). It is now evident that *p16<sup>Ink4a</sup>/MTS1/CDKN2A* alone, but not its close relative *p15<sup>Ink4b</sup>/MTS2/CDKN2B*, can sustain tumor-specific mutations in a large number of tumors. The *p16<sup>Ink4a</sup>/MTS1/CDKN2A* locus encodes two overlapping genes, each regulated by its own promoter. The transcript generated from the distal promoter encompasses exons 1 $\alpha$ -2-3 encoding p16. The transcript generated by the proximal promoter is formed by exons 1 $\beta$ -2-3 in a different reading frame and encodes a completely different protein, p19<sup>ARF</sup>. The N-terminal 64 amino acids of p19<sup>ARF</sup> are encoded by the unique exon 1 $\beta$  while the C-terminal 105 amino acids are encoded by an ARF in exon 2 (143). As discussed earlier, 19ARF overexpression induces p53-dependent growth arrest, plays a role in the regulation of p53, and is a bona fide tumor suppressor in mice (16,144,145).

Three major mechanisms of inactivation of the *INK4a* locus in human cancers are deletion of both alleles or deletion of one allele, and either intragenic mutation of the remaining allele or methylation of the remaining allele (146–150). Deletions remove both p16 and p19<sup>ARF</sup> (and occasionally p15), while intragenic point mutations that are frequent in the unique exon 1 $\alpha$  of *p16<sup>Ink4a</sup>/MTS1/CDKN2A* or in the common

exon 2 appear to inactivate p16 only function (151). Therefore, deletions and intragenic mutations must be functionally distinct.

Point mutations lead to the inactivation of the pRb pathway while deletions inactivate both the pRb and p53 pathways. One of the most striking differences between human tumors is the relative frequency of deletions and mutations in this locus.

Homozygous deletions appear to predominate in gliomas (57%), mesotheliomas (56%), leukemias (40%), nasopharyngeal carcinomas (42%), sarcomas (8%), ovarian carcinomas (16%), and bladder carcinomas (18%). by contrast, esophageal (30%) and biliary tract cancers (58%) sustain only intragenic point mutations. Both deletions and mutations have been detected in head-and-neck carcinomas (8% mutations, 6% deletions) and NSCLC (16% mutations, 14% deletions) (146–148,152). Ninety-eight percent of the pancreatic cancers have inactivated p16: 48% homozygous deletion, 34% hemizygous deletion and intragenic mutation, and 16% hemizygous deletion and methylation-mediated silencing (146–152). Neither deletion nor mutation is detected in breast cancers, neuroblastomas, colorectal tumors, and nonacute lymphocytic leukemia leukemias.

### **Mutually Exclusive Alterations of Cyclin D1, p16, and pRb**

The only known physiologic substrate of the CDK4/cyclin D1 kinase is pRb. Progression through G1-phase requires phosphorylation and inactivation of pRb by the CDK4/cyclin D1 kinase and liberation of the pRb-associated transcription factors (Fig. 4). The implication of this functional link is that perturbation in any of the genes involved in this pathway will likely have similar negative consequences. Evidence suggests that the CDK4/cyclin D1/p16/pRb pathway behaves as a single mutagenic target during tumorigenesis. Amplification or overexpression of *CCND1* and/or *CDK4* or *CDK4* mutation will promote pRb phosphorylation and inactivation, leading to unrestrained cell proliferation. Inactivation of p16 will have the same effect. In tumors with a mutated or deleted *Rb* gene, additional selection for alteration of the upstream genes is not necessary. Consequently, in *pRb*-negative cells, levels of cyclin D1 are reduced and levels of wild-type p16 are increased. *Rb*-positive cells frequently have *CDK4*-cyclin D1 amplification/overexpression and/or mutation of *p16<sup>Ink4a</sup>/MTS1/CDKN2A*. Mutually exclusive inactivation of *p16* or pRb has been observed in gliomas (128,133,135). Small-cell lung carcinomas generally express wild-type p16 and have mutated pRb, while NSCLCs show frequent loss of p16 and have wild-type pRB (153).

This inverse correlation also applies to *CDK4* amplifications vs *p16<sup>Ink4a</sup>/ME1/CDKN2A* inactivation in sarcomas and gliomas (128,133,135). In familial melanoma, approx 50% of the families have inactive alleles of *p16<sup>Ink4a</sup>/ME1/CDKN2A* while approx 5% have the *CDK4 R24C* mutation that disrupts the interaction of CDK4 with p16 (138). As more data become available, it is becoming evident that the CDK4/cyclinD1/p16/pRb pathway is deregulated in most human tumors, and the deregulation of this pathway is a common theme in tumor development.

### **Reduced Levels of p27 Protein in Human Tumors**

The *p27Kip1* gene is not mutated in most human cancers although the gene itself is localized to the chromosome band 12p13, a locus known to be altered in leukemias and mesotheliomas. Further analysis of these tumor samples did not reveal tumor-specific mutations in the coding region of the *p27Kip1* gene (154,155). However, p27 protein levels appear to be reduced in human tumors. More significantly, this reduction of p27

protein levels strongly correlates with tumor progression and poor survival in patients with breast, colon, or gastric carcinomas (156–159). In breast cancer samples, p27 consistently decreased with increasing tumor grade and is a strong predictor of reduced disease-free survival, and it appears to be an independent prognostic indicator (157,160). The value of p27 as a prognostic marker is even higher when it is used in combination with cyclin E. Combinatorial analysis of p27 and cyclin E expression levels showed that patients with low cyclin E and high p27 levels have a considerably longer survival rate than patients with high cyclin E and low p27Kip1 levels (158). In colorectal and gastric carcinomas, low p27 levels correlate significantly with poor survival (156,159). The subset of patients whose tumor lacked any detectable p27 exhibited a uniformly poor prognosis. In addition, in prostate adenocarcinomas, low p27 expression predicts poor disease-free survival and is an independent predictor of treatment failure after radical retropubic prostatectomy (161,162). Similarly, in Barrett's-associated adenocarcinomas of the esophagus, the loss of p27 is associated with parameters of aggressive behavior such as higher histologic grade, depth of invasion, presence of lymph node metastasis, and survival (163).

In normal cells, p27 regulates the progression from G1- into S-phase by binding to and inhibiting the activity of the CDK2–cyclin E complex (Fig. 9). In p27-negative tumors, there is an increase in cyclin A- and cyclin E-associated kinase activity but no significant correlation between p27 expression levels and proliferative status (156,157). These data may suggest an additional role for p27. For example, p27 has been shown to play a role in adhesion-dependent cell growth, and the loss of p27 may confer the ability to grow in an environment with altered ECM properties, thus facilitating metastasis (164,165). p27Kip1 may also regulate the exit from the cell cycle and initiation of cell differentiation. In this regard, it is noteworthy that in colon carcinomas the well- or moderately differentiated carcinomas had high p27 levels while the poorly differentiated carcinomas had lower expression of p27 (166).

These studies showed no correlation between *p27Kip1* mRNA and protein levels, suggesting that a posttranscriptional mechanism(s) is responsible for the reduction of p27 in tumor cells (156,157,163,166). p27 protein levels are regulated by the ubiquitin-mediated degradation of p27 by the proteasome (81). It has been suggested that the increased activity of a p27Kip1-specific degradative pathway may be responsible for the reduction of p27 in tumor cells (156). How specific this degradative pathway is remains controversial.

### **Alterations in Other Cell-Cycle Regulators**

*cyclin E* overexpression has been reported in a high proportion of breast cancers, but the *cyclin E* gene itself is amplified in a small number of cases (105,167–169). Together with p27, cyclin E overexpression has prognostic value for the outcome of the disease.

Although historically important, only one example exists of a human liver carcinoma in which the *cyclin A* gene was overexpressed as a result of hepatitis B virus integration (170,171). It is important to point out that both cyclin E- and cyclin A-associated kinase activity may become deregulated as the direct consequence of increased p27 degradation in human tumors.

Another mechanism that may lead to deregulated cyclin D-, cyclin E-, and cyclin A-associated kinase activity is overexpression of the putative activating phosphatases CDC25A and CDC25B. CDC25B mRNA is overexpressed in 32% of human breast can-

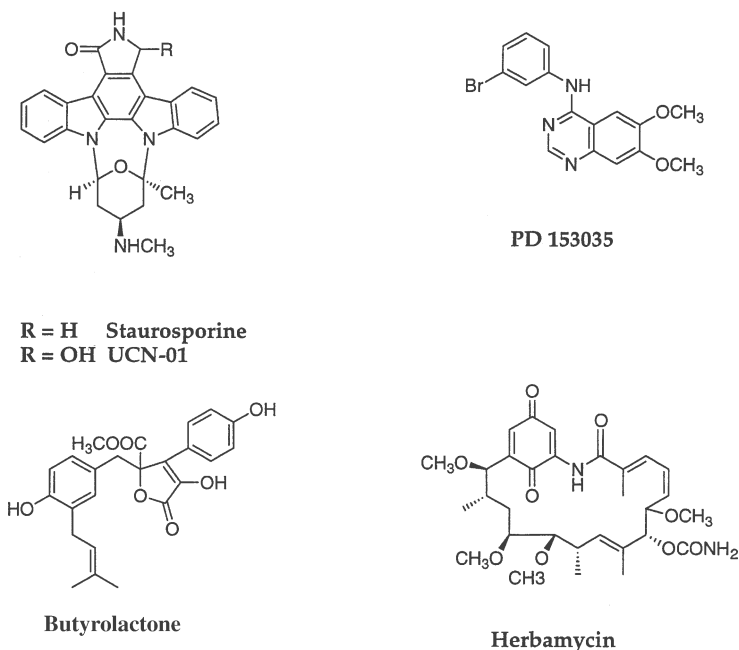


Fig. 10. Kinase inhibitors identified from screening.

cers, and its overexpression was most frequent in high histological grade cancers with poor prognosis, although the prognostic value of these findings is currently unclear (63,172,173).

### ***Inhibitors of CDK as Therapeutic Agents***

One of the reasons that the cell cycle is such a compelling drug discovery target is that it represents one of the most downstream signals that must be deranged before cell-cycle disruption. The central role of the p16/CDK4/cyclin D/pRb pathway supports this hypothesis (Fig. 4). Many technologies have been used as potential therapeutic options. Reports exist of gene therapy, protein therapy, antisense, and small molecule approaches to correct cell-cycle alterations.

### ***Small-Molecule Approaches***

CDKs have a central role in the initiation and orchestration of cell-cycle events. Several examples where alterations in these key CDKs lead to tumor development are known. Specifically, upregulation of CDKs has been linked to transformation. Considerable effort has been devoted, therefore, to developing inhibitors of these CDKs. CDK require that several biochemical steps be coordinated and occur in a linear sequence for these enzymes to function correctly. Each of these steps represents, in theory, a viable drug discovery target (174). Despite the wide range of possibilities, most of the effort has been devoted to finding inhibitors of the catalytic activity of CDK enzymes. This focus on the catalytic activity is driven by the recent success the pharmaceutical industry has enjoyed in finding specific inhibitors of various kinase enzymes. One company (Fig. 10) has reported on an epidermal growth factor receptor (EGFR) antagonist that has 5 pM activity with apparent exquisite selectivity (175). These inhibitors of kinase catalytic activity tend, in the main, to be ATP-competitive inhibitors.

ATP-competitive inhibitors pose two problems, both of which are related to specificity. The first relates to chemical specificity. Because a very large number of kinases are present in a cell, research groups screen against representatives of different kinase families. Because these assays are not comprehensive, it is very difficult to know whether the cellular effect or phenotype observed with a specific inhibitor is due to inhibition of the kinase of interest or some other totally unrelated kinase. An example of this relates to staurosporine and UCN-01; both are *bis*-indole compounds and have very closely related structures (Fig. 10). Both of these analog are broad-spectrum kinase inhibitors and show cell-growth inhibitory effects. In addition, both alter the ratio of phosphorylated and unphosphorylated pRb, but this ratio is different for the two compounds. The cellular effects observed for these two compounds may be due to inhibition of other unrelated kinases and not inhibition of CDKs themselves, or a combination of CDK inhibition with inhibition of a second kinase (176).

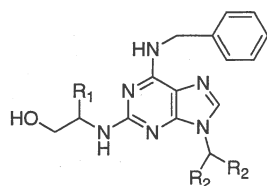
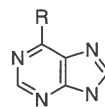
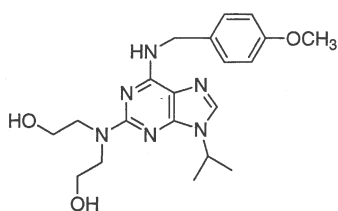
This lack of biologic specificity can be very important, especially in tracking side effects of various compounds. Cellular markers that follow the mechanism of action of the specific kinases of interest do provide data that give some insight into the phenotype. The specific phosphorylation sites on the Rb protein modified by individual CDKs are known (23). The CDK4/cyclin D enzyme preferentially phosphorylates pRb on S-795, while CDK2–cyclin E and A phosphorylates on S-821 (23). Phosphorylation at these positions can be taken as a qualitative indication that some part of the biologic effect is due to inhibition of CDKs. Given their role in driving cell-cycle progression, compounds that inhibit all CDKs would be expected to induce both G1 and G2 arrests.

### General Classes of CKIs

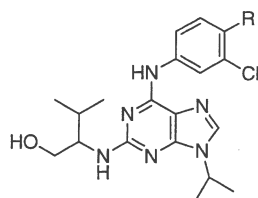
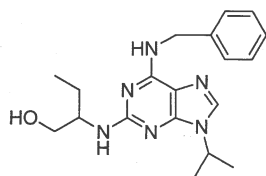
The potential CKIs can come from natural product screening, compound libraries, and combinatorial chemistry. Any kinase inhibitor from an unrelated kinase program can also be used as a starting point for a potential CKI. Several classes of kinase inhibitors such as staurosporine, naphthalene sulfonamides, isoquinoline derivatives, and sphingosine have been reported (177,178). A number of these have been tested against CDK and shown to be active (179,180). In general, these compounds display broad specificity (177,178). Figures 10 and 11 show some of the initial lead structures from various screening approaches that have been identified as CKIs.

The natural product butyrolactone was discovered by screening against murine CDK1–cyclin B and is an ATP-competitive inhibitor (Fig. 10) (181–183). It shows some selectivity among CDKs, being more potent against CDK1 and CDK2 and showing little effect against CDK4/cyclin D (Table 1). It was shown to inhibit Rb phosphorylation in vitro and in vivo. Butyrolactone shows activity in WI38 cells and causes a G1- to S-phase arrest. The compound also inhibited histone–H1 phosphorylation and caused concomitant G2- to M-phase arrest. This pattern of G1- to S-phase and G2- to M-phase arrest will be seen many times with inhibitors that are active against several of the different CDK enzymes. Herbamycin (Fig. 10) has been shown to decrease amounts of CDK6 but has no effect on the closely related CDK4 complex or CDK2 (184). This selectivity is very unusual. Because CDKs are so well regulated, this compound could well be involved in some pathway that controls CDK6 concentrations without exerting a direct effect on CDK6 itself.

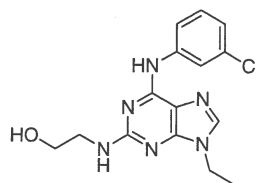
By contrast, other well-known inhibitors of kinases such as staurosporine (Fig. 10) tend to inhibit all kinases equally well. For CDK selectivity, this “pan-kinase” activity requires that the chemists design away off-target kinase activity while keeping the CDK

R<sub>1</sub> = R<sub>2</sub> = H OlomoucineR<sub>1</sub> = C<sub>2</sub>H<sub>5</sub> R<sub>2</sub> = CH<sub>3</sub>  
RoscovitineR = NH-CH<sub>2</sub>-CH=C(CH<sub>3</sub>)<sub>2</sub>  
IsopentenyladenineR = N(CH<sub>3</sub>)<sub>2</sub> 6-DMAP

CVT 313

R = H Purvalanol A  
R = COOH Purvalanol B

Roscovitine



Structure 1

Fig. 11. Purine CKIs.

**Table 1**  
**CDK Specificity of Known Kinase Inhibitors**

Target	Staurosporine	UCN-01	Butyrolactone	Flavopiridol	Olomoucine	Roscovitine
CDK1	0.003–0.009	0.031	0.60	0.40	7.00	0.65
CDK2	0.007	0.030	1.50	0.40	7.00	0.70
CDK4	<10.0	0.032	>1000	0.40	>1000	>100
PKA	0.008	—	200	145	>2000	>1000
PKC	0.005	0.007	160	—	>1000	>100

**Table 2**  
**Purine CKIs**

Enzyme/Cpd	CVT-313	Purvalanol A	Purvalanol B	Roscovitine	Structure 1
CDK2/E	0.5	35.0	9.0	0.70	—
CDK1/B	4.2	4.0	6.0	0.25	0.08
CDK4/D <sub>1</sub>	215	850	>10,000	>100	—
PKA	1250	9000	3800	>1000	180
PKC	1250 >10,000	>100,000	>100	31.5	
Mech'n	ATP	ATP	ATP	ATP	

activity. This result can be achieved; the purine analogs isopentenyladenine and 6-dimethylaminopurine (6-DMAP) (Fig. 11) are nonselective kinase inhibitors, but olomoucine and roscovitine, which are derived from these leads, do offer more selectivity (Table 2).

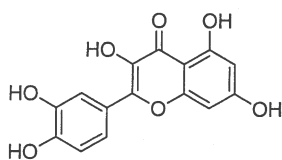
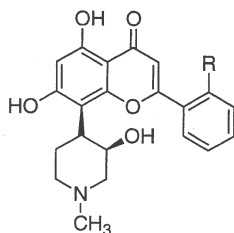
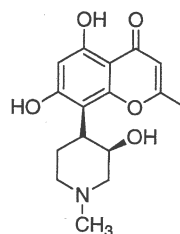
The final class of inhibitors covered is flavones. This class of compounds is known to inhibit receptor protein kinases (177). In routine testing of compounds in the National Cancer Institute (NCI) panel of human tumor cell lines (leukemia, NSCLC, colon, renal, prostate, and breast cancer cell lines) (185), L 86-8275 (flavopiridol) was shown to be active in cells (Fig. 12). When the mechanism of action of this flavone was examined, it transpired that this compound inhibited CDKs (186). L 86-8275 is currently in phase 2 clinical trials, either by itself or in combination with conventional cytotoxic drugs.

### **Purines as CKIs**

Isopentenyladenine and 6-DMAP are nonselective CKIs (Fig. 11). Examination of the structures of these two compounds shows the relation to the structure of adenine. Several purine analogs including isopentenyladenine and 6-DMAP have been shown to be ATP-competitive kinase inhibitors (187). Screening has led to the discovery of olomoucine as a reasonably selective CDK inhibitor (Table 2). Olomoucine is a selective inhibitor for CDK2 and CDK1 and shows little activity against CDK4 or other protein kinases such as protein kinase A (PKA) and PKC (Table 2). An understanding of the reasons for this selectivity may allow preparation of CDK-specific inhibitors. A number of the CDK and cyclin subunits have been crystallized individually or as complexes. Various groups have crystallized several of the small-molecular-weight inhibitors in the active site of the CDK subunit. Among the bound complexes is one with olomoucine, isopentenyladenine, flavopiridol, and purvalanol B in the active site of CDK2. It is important to note that all the cocrystals, to date, have been with the monomeric (and thus inactive) CDK subunit.

Examination of the cocrystals of the CDK subunit with ATP, olomoucine, and isopentenyladenine offers insights into the observed selectivity (188). The three purine rings in these three analogs bind in the same portion of the ATP-binding cleft, but their three orientations are different. In ATP the N-6 amino group is pointed toward the deepest part of the ATP-binding pocket. The larger N-6 substituents in olomoucine and isopentenyladenine (Fig. 11) make it sterically impossible to sit in exactly the same manner as ATP. In these two analogs, the N-6 groups occupy the space occupied by the ATP ribose and phosphate groups. With isopentenyladenine, the bulky N-6-isopentenyl



**Quercetin****Genestein**

R = H L 86-8275

R = Cl L 86-8275 Flavopiridol

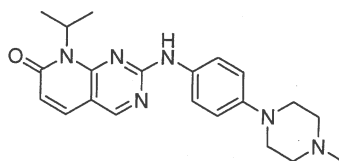
**Rohitukine****PD-172803**

Fig. 12. Flavone CKIs.

group takes the position of the ribose ring of ATP, and with olomoucine, this space is occupied by the N-2-hydroxyethyl group. The selectivity observed with olomoucine is explained by the position of the N-6-benzyl group. In olomoucine, this group binds toward the outside of the ATP-binding pocket in a region not occupied in the ATP-CDK2 complex or the isopentenyladenine-CDK2 complex.

One advantage of these purine derivatives is their synthetic accessibility. Much is known about basic nucleic acid chemistry, and the purine scaffold is amenable to combinatorial chemistry approaches. The purine nucleus has three positions amenable to combinatorial chemistry approaches; all three positions have been studied in both combinatorial and traditional approaches (189–194) (Fig. 11). The structure-activity relationships from all these and related approaches suggest that the substituent on N-9 should be a small alkyl group; the substituent at the 6-position is optimal if it is an aryl, aralkyl, or substituted aryl. The C2 position is best as a substituted ethanolamine group.

Peptide 1 : K A C R R L F G P V D S E Q L S R D C D  
 Peptide 2 : K R R Q T S M T D F Y H S K R R L I F S  
 Peptide 3 : K R R L I F S K  
 Peptide 4 : A C R R L F G P V D S E  
 Peptide 5 : P V K R R L D L  
 Peptide 6 : D A A R E G F L D T L V V L H R A G A R  
 Peptide 7 : F L D T L V V L H R

Fig. 13. Non-ATP-competitive peptide CKIs. Letters are symbols for amino acids.

One of these combinatorial studies has led to the synthesis of purvalanol B, one of the more selective CDK2 antagonists (Fig. 11) (195). This compound is very closely related to olomoucine and shows similar selectivity (Table 2). An X-ray structure of purvalanol B with CDK2 shows that the selectivity of this compound arises from the unique interactions of the N-6 group substituent. CVT-313 also shows selectivity for CDK2 compared with other kinases, as do roscovitine and structure 1 (Fig. 11, Table 2). The cellular effects of CVT-313 (196), olomoucine (197), and purvalanol A have been examined. CVT-313 and olomoucine both show a G1- to S-phase arrest at lower concentrations and both a G1- to S-phase and G2- to M-phase arrest at higher concentrations. No cell-cycle arrest data are available for purvalanol A. In terms of cellular potency, CVT-313 has an average IC<sub>50</sub> (50% inhibitory concentration) for growth inhibition ranging from 1.5 to 2.0  $\mu$ M in nine cell lines (196). Purvalanol A and olomoucine have been tested against the NCI panel of human tumor cell lines (leukemia, NSCLC, colon, renal, prostate, and breast cancer cell lines) (185) and have an average IC<sub>50</sub> of 2 and 60  $\mu$ M, respectively. CVT-313 has been tested in a rat carotid artery model of restenosis and results show that at 1.25 mg/kg for 15 min under pressure, the compound can reduce neointima formation by 80%.

### Flavones as CDKIs

A natural product screen for anti-inflammatory compounds led to the discovery of rohitukine (Fig. 12). Further testing of rohitukine and its analogs for inhibition of the EGFR and for cytotoxicity on selected tumor cell lines showed flavopiridol to be the derivative with the strongest inhibitory and cytotoxic activity in vitro (Fig. 13). Flavopiridol exhibited in vivo growth inhibition of human tumors xenografted onto *nu/nu* mice (198). The average potency of flavopiridol in the NCI tumor cell line panel was 66 nM (186). These cell experiments suggest that the effects of flavopiridol are far greater on tumor cells than normal bone marrow cells.

The antitumor activity of flavopiridol in vitro and in cells was much higher than would be predicted from its activity against known kinases. The cell-cycle effects of flavopiridol show that the compound can cause both G1 and G2 arrest in asynchronous cells (199). Testing against the individual kinases showed that inhibition of CDK1/cyclin B can account for the G2 arrest phenomena observed (200). The progression through G1- to S-phase is controlled by both CDK4 and CDK2. Flavopiridol inhibits both these enzymes (Table 3), which could account for the observed G1 arrest (201). The structural activity relationships around Flavopiridol has been reported elsewhere (186).

**Table 3**  
**Subacute Toxicities of Flavopiridol**

Species	Application	Dose schedule	Results	Conclusions
Rats (Fischer 344) <sup>a</sup> (10/sex/dose)	9 × iv (every 8 h for 72 h)	0.5; 1; 2 mg/(kg-injection) (1.5; 3; 6 mg/kg/d)	≥1.5 mg/kg/d ≥3.0 mg/kg/d  Soft or loose stools; reversible atrophy of thymus splenic lymphoid follicle, and bone marrow; decrease of RBCs, Hb, leukocytes, platelets Lethal (1/10)	Maximum tolerated dose: 1 mg/(kg-injection) (3 mg/kg/d to 18 mg/m <sup>2</sup> /d)
Rats (Fischer 344) <sup>a</sup> (5 male/dose)	9 × iv (every 8 h for 72 h)	2; 4; 6; 10 mg/(kg-injection) (6; 12; 18; 30 mg/kg/d)	≥6.0 mg/kg/d ≥6.0 mg/kg/d  Soft or loose stools; reversible atrophy of thymus, splenic lymphoid follicle, and bone marrow; decrease of RBCs, Hb, leukocytes, platelets Lethal (5/5)	—
Dogs (Beagle) <sup>a</sup> (2/sex/dose)	1 × 72 h continuous infusion	0.8; 1.3 mg/kg/d	0.8 mg/kg/d 1.3 mg/kg/d  No sign of toxicity; increased soft stool; decrease in food consumption; increase in ck and ap; multifocal congestion of mucosal vasculature of gut	Tolerated dose: 0.8 mg/kg/d (16 mg/m <sup>2</sup> /d)
Dogs (Beagle) <sup>b</sup> (1 male/dose)	72-h infusion	1.9; 2.8 mg/kg/d	≥2.8 mg/kg/d Lethal	Toxic dose low: 1.3 mg/kg/d (26 mg/m <sup>2</sup> /d)

<sup>a</sup> Ameson D et al. *Proc Am Assoc Cancer Res.* 1995; 36: abstract 366.

<sup>b</sup> Southern Research Institute (Birmingham, AL.) sponsored by the NCI-DTP.  
iv, intravenous; RBCs, red blood cells; Hb, hemoglobin.

The X-ray structure of the flavopiridol analog L 86-8275 (Fig. 12) bound in CDK2 has been reported. As with the earlier analogs discussed, flavopiridol occupies the ATP-binding pocket with the benzopyran ring occupying approximately the same space as the purine ring of ATP. This benzopyran ring is rotated to accommodate the molecule. The aromatic ring is pointed to an area of the ATP-binding pocket not normally used by ATP. The appended nitrogen containing the piperidine ring occupies the phosphate-binding region of the binding pocket (202,203). Flavopiridol has been tested in various preclinical and pharmacokinetic models. The dose-limiting toxicities are shown in Table 3. It has entered phase 2 trials using a 72-h continuous-infusion dosing schedule at 50 mg/m<sup>2</sup>/d. Synergy studies are underway (204).

All the compounds discussed have been considered as “CDK2-selective” inhibitors. To date, very few CDK4-selective compounds have been disclosed. Parke-Davis has reported that PD-172803 is 10-fold selective for CDK4 (Fig. 12), not to be confused with another of its compounds (PD 180970) that blocks constitutive activation of STAT-5 (signal transducers and activators of transcription-5), one of the critical transcription factors downstream of Bcr-Abl in chronic myeloid leukemia.

### **Non-ATP-Based Approaches**

Finding compounds that inhibit the activity of CDK by a non-ATP-competitive mechanism has the advantage of obviating chemical and biologic specificity issues. Several groups have examined proteins that interact with CDK to ascertain whether these interactions can be copied with small molecules.

A systematic study of p21 protein was undertaken by several groups. These studies identified two domains responsible for binding to CDK and the cyclin subunit. In studies using overlapping peptides spanning the whole of p21, two 20-amino-acids peptides (peptides 1 and 2; Fig. 13) were reported to bind and inhibit the catalytic activity of both CDK4/cyclin D and CDK2/cyclin A or E (205). One of 20-mer was truncated to an 8-mer (peptide 3; Fig. 13) that still showed activity in the relevant assays. The two interacting domains (peptides 3–5) were identified by a separate group using a different starting point (206).

A similar approach has been described for p16 (207,208). Again, a 20-amino-acid peptide (peptide 6; Fig. 13) has been identified as being inhibitory to CDK4/cyclin D. This peptide spans the 84–103 stretch of p16. An independent analysis identified this region as necessary for p16 function. An Ala scan was conducted and a truncated peptide of 10 amino acids (peptide 7; Fig. 13) was shown to be active against CDK4/cyclin D. Peptides from p16 do not contain the cyclin-binding motif identified in the other approaches.

In an interesting proof-of-principle series of experiments, the 16-amino-acid sequence from *Antennapedia* responsible for the internalization across the membrane (called Penetrin) was linked to peptides 2, 3, 6, and 7 (205,207,208). The internalization sequence should enable these peptides to cross the cell membrane and exert a cellular effect. The chimeric peptide produced from peptides 2 (M92A) and 3 with Penetrin was tested on HaCaT cells. In both cases, G1 arrest and a reduction in pRb occurred, suggesting that the cellular effect is by the expected mechanism. The chimera with peptide 2 is effective at 25  $\mu$ M, while the peptide 3 chimera shows comparable effects at 50  $\mu$ M. The p16-derived chimeric peptides were tested in a similar manner. Peptide 6 (D92A mutation) linked to Penetrin showed good activity at 12  $\mu$ M; it efficiently blocks serum-deprived HaCaT cells from entering S-phase and causes

near-complete G1 arrest. This effect lasts 36–48 h. This chimeric peptide was also tested in six cell lines at 20  $\mu\text{M}$ . It again blocked S-phase entry in four of the cell lines. This chimeric peptide had no effect on a pRb-negative cell line, suggesting that the phenotype being observed is mechanism related. Peptide 7 linked to Penetrin also blocks cell-cycle progression.

A yeast two-hybrid approach has been developed to identify random peptides, called aptamers, that are dominant inhibitors of protein function (209). The aptamers are *Escherichia coli* thioredoxin molecules that display 20-amino-acid long random peptides in their active site loops. Aptamer libraries are used in the two-hybrid system to identify peptides that bind to a target protein. Aptamers that bind specifically to the target are tested for their ability to interfere with the function of the target protein. For example, aptamers have been identified that are capable of binding to CDK2 tightly (dissociation constant as low as 38 nM) and selectively (most of them bind only to CDK2 and not to other CDK family members). Several inhibit CDK2 activity in vitro. One of them (pep8) binds to CDK2 near its active site and selectively inhibits the phosphorylation of histone H1 ( $\text{IC}_{50}$ : 5 nM) but not pRb by CDK2/cyclin E. This distinct substrate specificity is not observed with natural inhibitors. The mode of inhibition is competitive, suggesting that pep8 interferes with the interaction of CDK2 with histone H1 but not pRb. Expression of pep8 in cells blocks the cell cycle at the G1- to S-phase transition, possibly because pep8 interferes with the phosphorylation of substrates required to pass through G1 (210). These aptamers may help to dissect the functions of different CDK2 substrates and aid the development of highly specific, nonpeptidic inhibitors of CDK2.

## Gene Therapy Applications

The natural CKIs are highly potent and specific inhibitors of CDKs. The overproduction of CKIs in both normal and tumor cells leads to cell-cycle arrest and inhibition of cell proliferation. These observations suggest a use for CKIs as cytostatic agents for the treatment of proliferative diseases. In this approach, the gene encoding a CKI protein is linked to a vector DNA that facilitates the uptake and high-level expression of the transgene. The most popular vector system used for the delivery of CKI is based on human adenoviruses (Ads). These replication-deficient Ad vectors infect a variety of cell types both in vitro and in vivo and can program high levels of transgene expression for several weeks after infection.

Ad vectors that direct the expression of p16, p21, and p27 (Ad-p16, Ad-p21, and Ad-p27) have been constructed and tested both in tissue culture and in animal models. As expected, the overexpression of CKI inhibits CDK activity, G1 arrest, and cell proliferation in both normal and tumor cells (211,212). Ad-p16 inhibits the proliferation of cells that express wild-type pRb (*Rb+*), and has little or no effect on the proliferation of cells with mutant pRb (*Rb-*) (213). Ad-p21 and Ad-p27 constructs inhibit both *Rb+* and *Rb-* cells equally well. In addition to their cytostatic effect, a varying degree of cytotoxicity is associated with Ad-CKI infections. It has been shown that p27 overexpression in normal and cancer cells induces apoptosis (214–216), although the apoptotic effect may also be due to the collaboration of p27 and the *E4* gene product of the Ad vector.

Intratumoral injections of Ad-CKI vectors into tumor xenografts inhibit tumor growth. In accordance with its cytotoxic effect, however, Ad-p27 injections into breast xenografts cause tumor regression (217). The codelivery of p16 and p53, but not p16 or

p53 alone, into tumor cells induces apoptosis and tumor regression in xenografts (218). These results suggest that an optimal antitumor effect may be achieved only with Ad-CKI in intratumoral injections when the cytostatic effect of the inhibitors is combined with cytotoxicity and apoptosis through the cooperation of CKIs with another gene product(s), or possibly with chemotherapeutic agent(s). It is feasible, however, to deliver the vector constructs directly to endothelial cells in the tumor vasculature, inhibiting endothelial cell proliferation and migration and tumor-specific angiogenesis. In this scenario, the disruption of the tumor vasculature by Ad-CKI infection would lead to tumor cell apoptosis owing to hypoxia, starvation, and increased concentration of toxic metabolites in the tumor.

### Protein Therapy Applications

The inhibitory effects of CKIs depend on their relative abundance to their respective CDK/cyclin targets. The concentration of CKI must exceed that of the target CDK to inhibit the target's activity completely. As discussed earlier, this can be achieved in vivo by the ectopic overexpression of CKI using gene therapy. Theoretically, the cellular concentration of CKI can be increased by direct delivery of the inhibitor proteins. The inherent problem with this approach is that CKIs are intracellular proteins and exogenous CKIs are unable to cross the cell membrane and localize to the nucleus. Fortunately, a number of proteins have been described with the demonstrated ability to penetrate cell membranes and carry covalently linked cargo proteins inside the cell. These proteins include the products of two viral genes, the HIV-1 TAT (219) and HSV-1 VP22 (220) proteins, as well as peptides derived from the *Drosophila melanogaster antennapedia* (221) and the human fibroblast growth factor (FGF) proteins (222). These polypeptides can function as "delivery tags" facilitating the cellular uptake of cargo proteins from the extracellular space. To use these delivery tag-inhibitor fusions for the treatment of cancer and other proliferative diseases, the proteins must be delivered locally to the site of the diseased tissue in sustained-release formulations.

Penetrin (a 16-amino-acid long peptide of *D. melanogaster antennapedia* protein) can mediate the delivery of p16- and p21-derived peptides into cells and inhibit cell proliferation, although at relatively high concentrations ( $IC_{50}$ : 10–50 mM). Different size peptides of the HIV-1 TAT protein have been used to deliver p16 and p27 into normal and tumor cells. These experiments have identified two short peptides of TAT (amino acids 48–60 and 47–58) that when fused to p16 or p27 efficiently mediate their cellular uptake and nuclear localization. These exogenously added TAT-p16 and TAT-p27 proteins are potent inhibitors of cell proliferation. For example, the  $IC_{50}$  of the various TAT-p27 fusion proteins is in the 0.8–5 mM range when added to human primary coronary artery smooth muscle cells (223).

### Antisense Approaches

Because CDKs and cyclins are essential for cell proliferation, they are popular targets for approaches using antisense oligonucleotides. Antisense oligonucleotides are short (15–25 nucleotides) nucleic acid segments that are complementary to the target mRNA. Inside the cell, antisense oligonucleotides hybridize to the complementary mRNA according to the Watson–Crick pairing rules. It is thought that this heteroduplex is degraded by RNase H leading to the inhibition of gene function (224).

As expected, the ablation of CDK or cyclin functions inhibits proliferation of all cell types (with the exception of cyclin D1 inhibition in *Rb*– cells). Inhibition of p27 with

an antisense oligonucleotide in a 3D culture of tumor cells sensitizes slowly proliferating tumors to chemotherapeutic agents and to radiation by increasing cell proliferation and reducing intercellular adhesion. This observation suggests that p27 antagonists potentially may be useful chemosensitizers in conjunction with traditional chemotherapeutic agents (164).

The use of antisense oligonucleotides as therapies against disease-causing genes is a very attractive idea, as theoretically a 15-nucleotide oligonucleotide has the base-pairing specificity to interact with only one target within the human genome. Unfortunately, several issues hinder the development of antisense-based therapies including the nonspecific and nonantisense mechanism of action, intracellular stability, affinity to the target sequence, cell permeation, and delivery of the oligonucleotides. Significant advances must be made in these areas before this approach lives up to its potential.

### Early Clinical Trial Results With Synthetic Inhibitors

Staurosporine and its related analog UCN-01 are broad-spectrum kinase inhibitors and show cell-growth inhibitory effects in multiple *in vitro* models. Treatment of human tumor cells with UCN-01 dephosphorylates Rb and CDK2 proteins and increases the amount of CKIs, p21 and p27 (225), leading to arrest in G1. Unfortunately, besides the problem of chemical specificity, UCN-01 in humans has shown an extremely long half-life (>24 h), and a low systemic clearance due to binding to several serum proteins, in particular, human  $\alpha$ -1 acid glycoprotein (AGP) (226). An early phase 1 clinical report observed toxicities such as nausea and vomiting; hyperglycemia; and, more dangerously, pulmonary toxicities with the risk of severe hypoxia (227). In that study, UCN-01 was given by 72-h continuous iv infusion every 2 wk. Median salivary-free UCN-01 concentrations at maximum tolerated dose was 45 nM, a concentration associated with G2-checkpoint arrest in *in vitro* models. In another phase 1 study with the same drug, administering it by 3-h infusion, the toxicities appeared to be similar but were less severe (228). The peak plasma concentrations, however, tended to be saturable because of binding to AGP.

Flavopiridol was the first CKI to enter clinical trials. In 1998 (229), a phase 1 trial of continuous-infusion flavopiridol was reported by NCI. Seventy-six patients with refractory malignancies and disease progression were treated with flavopiridol given as a 72-h infusion every 2 wk. Diarrhea (largely controllable), hypotension, and a “pro-inflammatory syndrome” with alterations in acute-phase reactants were reported as side effects of this drug. Concentrations of flavopiridol (200–400 nM) needed for CKIs in preclinical models were achieved safely, but unexpected postinfusion peaks of serum concentration of the drug were observed in 30% of cases. A 2000 University of Chicago study (230), demonstrated that in patients with kidney cancer receiving the drug by continuous infusion over 72-h (at 50 mg/m<sup>2</sup>/d) every 2 wk, there was no efficacy in this cancer type, and patients experienced other adverse effects such as vascular thrombotic events, including pulmonary emboli and myocardial infarction. Two other studies were reported. A phase 2 study of flavopiridol, administered as in the previous study, in patients with metastatic gastric cancer failed to show any significant clinical activity (231) and confirmed that the drug caused more fatigue and vascular thrombosis than anticipated from the phase 1 trials. In the second study (232), a combination of the conventional cytotoxic paclitaxel, as either a 24- or 3-h infusion, followed by flavopiridol administered by a 24-h infusion at 70 mg/m<sup>2</sup> on d 2 produced manageable toxicities and promising clinical activity in patients with incurable esophageal, lung,

and prostatic cancers. Paclitaxel, a taxane derivative from *Taxus brevifolia*, is known to bind to  $\beta$ -tubulins, causing disruption of the cytoskeleton, but it can also induce the expression of p53 and p21, by a Raf-dependent mechanism (233). It is possible to speculate that agents that inhibit CDKs, but that can also increase the expression of p53 will show increased cytotoxic activity.

Roscovitin is a synthetic trisubstituted purine that is thought to act primarily through inhibition of CDK2, and subsequent inhibition of phosphorylation of pRb and cell-cycle arrest. CYC202 is a formulation of the enantiomerically pure R-isomer of roscovitin and has the greatest potency for the CDK2/cyclin E complex. A dose-finding phase 1 study in patients with refractory malignancies has recently been reported in abstract form (234), and the drug showed few toxicities at the doses administered, with rapid absorption and rapid plasma clearance.

Identification of novel purine and pyrimidine CKIs with distinct molecular interactions and tumor cell growth inhibition profiles (235,236) is in progress, and new molecules are entering clinical development. Their potential clinical applications include not only cancer, but also other proliferative conditions such as atherosclerosis and restenosis of stents or coronary bypass grafts, as well as some proliferative glomerulonephritis and autoimmune conditions.

## Drugs Acting at Transcriptional Level

A common finding in most human cancers is the underexpression of CKIs, as a result of genetic and epigenetic alterations in cancer cells. The best-studied epigenetic causes of CKI underexpression are hypermethylation of the promoter gene sequences, and deacetylation of nucleosome-related histones.

### DNA Methyltransferase Inhibitors

Methylation of DNA serves as an epigenetic method of modulating gene expression, and hypermethylation has been demonstrated to silence a number of TSGs, including most CKIs (16). Epigenetic mechanisms of carcinogenesis are becoming clearer. Recent findings indicate that aberrant methylation can be detected in the smoking-damaged bronchial epithelium from cancer-free heavy smokers, suggesting that it as an ideal candidate biomarker for lung cancer risk assessment, and potentially useful for the monitoring of chemoprevention trials (237). Twelve of 12 lung cancer cell lines lacking a deletion or critical mutation in *Rb* or *CDKN2A*, which codes for the CKI p16<sup>INK4A</sup> protein, were found to be methylated at exon 1 of *CDKN2A* (238). Other studies have shown that underexpression of *CDKN2A* is frequently seen in early preinvasive lesions of squamous cell carcinoma of the lung and cervix (239).

While at least three functional DNA methyltransferases have been identified, the most abundant is DNMT1, which is responsible for methylation during DNA replication, and localizes at replication foci, at least in part by interacting with PCNA, a subunit of DNA polymerase *delta* that can bind to the CKI p21 protein. The enzyme DNMT1 catalyzes the covalent addition of a methyl group from a donor *S*-adenosyl-Met to the 5' position of cytosine, predominantly within the CpG dinucleotides in highly repeated transposable elements. These elements are termed *parasitic* due to their resemblance to viral DNA, and their ability to move between different chromosomal sites. During embryogenesis, there is an initial generalized demethylation of DNA, followed by a specific adult pattern of methylation. While approx 50% of the 5' promoter proximal elements contain CpG islands, they are not usually methylated in nor-



mal tissues. Cancers display a particular pattern of methylation, however. Overall, they are hypomethylated, but they do have specific regions of hypermethylation, often associated with regions rich in TSGs.

Several molecular variations of deoxycytidine have been developed to inhibit DNMT1, each modified at position 5' of the pyrimidine ring, and have been reviewed (240). Four agents have been used clinically: 5-azacytidine (azacitidine), 5-aza-2'-deoxycytidine (decitabine), 1- $\beta$ -D-arabinofuranosyl-5-azacytosine (fazarabine), and dihydro-5-azacytidine. These agents are not new. Some are almost 40 yr old, but other analogs such as cytosine arabinoside and gemcitabine (already used in the clinic as anticancer agents) do not inhibit methylation of DNA.

Because methylation of CpG islands in promoter proximal regions is uncommon in normal cells, normal gene expression should be largely unaffected by these agents. In some leukemic cells, however, decitabine increases expression of the multiple drug resistance *MDR1* gene product, which could be clinically counterproductive (241). Some studies with these inhibitors were done in the 1980s, such as studies with decitabine (242), and others have been completed more recently (243). In general, response rates to these agents in solid tumors have been rather low, but more promising results have been obtained in hematologic malignancies. Toxicities have been similar to conventional cytotoxics: granulocytopenia, thrombocytopenia, alopecia, nausea, vomiting, and diarrhea. The reason that these drugs have not yet translated into better clinical outcomes, in spite of sound biologic basis for their activity in human malignancies, is not clear.

### **Histone Acetyltransferases and Deacetylases**

Methylation of DNA induces recruitment of histone deacetylase (HDAC), which inhibits transcription, and agents have been devised that inhibit this deacetylase (244). The combination of a DNMT inhibitor and an h HDAC inhibitor might be a logical way to activate TSG expression (e.g., CKI), and a number of preclinical studies are in progress.

Chromatin is no longer considered a passive scaffolding for nuclear DNA. At the heart of chromatin's structure is the nucleosome, a complex of DNA wound around an octamer containing two molecules each of histone proteins H2A, H2B, H3, and H4 (245). Histones contain two domains: the protein's amino-terminal tails, which protrude from the nucleosome core, rich in charged amino acids such as Lys and Arg residues; and the globular histone core domain, mainly responsible for the histone:histone interactions involved in nucleosome formation.

Several posttranslational modifications occur in the histone tail domain, including acetylation, phosphorylation, ribosylation, methylation, ubiquitinylation, and glycosylation. The most well studied is the acetylation of histone tail domains on specific Lys residues. The steady-state levels of histone acetylation in the cell are maintained by a balance between the action of histone acetyltransferases (HATs) and HDACs.

Several methodological breakthroughs have allowed a better understanding of this process (246,247). Tightly compacted nucleosomes forbid general transcription factors, such as TFIID and RNA polymerase II holoenzyme, from interacting with promoter sequences of specific genes. It is becoming clear that many transcriptional activators actually direct two types of chromatin remodeling enzymes to specific promoters: an ATP-dependent remodeling enzyme and an HAT (248).

The field of chromatin research was revamped in 1996 with publication of evidence that the yeast Gcn5p protein, known to positively regulate gene transcription, was actually an HAT (249). This acetylation may induce a conformational change that weakens nucleosomal DNA:histone interactions, making the DNA more accessible to transcription factors. Many other transcriptional coactivators have been found to contain HAT activity. Other studies have identified several HDACs (including the mammalian HDAC1–8), as well as large multiprotein complexes, including the so-called RbAp-46 and -48 (retinoblastoma- and histone-binding proteins), that can undo histone acetylation and modify gene expression. The existence of a histone code whereby nonhistone proteins read the patterned display of various modifications on one or more histone tails resulting in the regulation of downstream transcriptional events has been proposed (250).

Considering all this new knowledge, it is no surprise that HAT and HDAC have become potential targets for cancer therapy (251). They appear to act as both oncogenes and tumor suppressors, depending on the genetic and epigenetic contexts. One interesting example is the variety of chromosome translocations, often an undesired consequence of previous cytotoxic chemotherapy itself, that can affect the mixed lineage leukemia (MLL) locus. Thus, an MLL–CBP fusion protein maintains the HAT activity of CBP, and it can be highly oncogenic, as the result of specific targeting of the fusion protein to certain genes (252).

The field of HAT- and HDAC-modulating drugs is promising but highly complex. To achieve selectivity, HAT must be highly targeted to specific genes, and this targeting depends on several structural protein motifs such as bromo domains and PHD fingers.

Allosteric inhibitors might be more selective and clinically useful than active site inhibitors. Traditionally, chemists have developed suitable pharmacologic compounds to interfere with active sites and substrate binding, rather than to interfere with the allosteric properties of a regulatory protein or protein complex. Even modern automated or semiautomated high-throughput screens are usually designed for compounds affecting enzyme activity, rather than for altering protein–protein interactions. This situation is a major challenge for the field of new anticancer drug development. Learning how to modify the allosteric properties of oncogenic proteins should become an important aspect of anticancer drug research.

### **Inhibition of the Ubiquitin-Proteasome Pathways**

Another research area that has evolved fairly rapidly in recent years is that of the so-called ubiquitin-proteasome pathways, involved in the processes of selective intracellular protein degradation. The intracellular levels of cyclins are strictly controlled and seem to be crucial for the normal functioning of several regulatory cell-cycle checkpoints. Indeed, the activity of cyclins appears to be mainly regulated by cyclin levels, rather than by intrinsic molecular changes. This situation does not mean, however, that cyclins are only regulated by their intracellular levels, and more research is needed to better define cyclin structure/function relationships in response to certain posttranslational modifications. CKI protein levels are strictly controlled throughout the cell cycle, and their degradation is dependent on the ubiquitin–proteasome pathways, too. At least theoretically, selective degradation of cyclins (that often act as oncogenes) but selective inhibition of degradation of CKIs (such as p16 or p27, which act as TSGs) would provide oncologists with new and powerful tools to fight cancer.

The overall regulatory process is rather complex. For example, in normal proliferating cells, the CKI p27 is actively degraded. The ubiquitin-conjugating enzyme Cdc34, an ubiquitin ligase (formed by at least four subunits: the F-box protein Skp2, Skp1, Cul1, and Roc1), and Cks1 are required for the transfer of ubiquitin to phosphorylated p27. In a self-amplification process, CDK phosphorylates p27 on Thr187, allowing p27 recognition by the Skp2 subunit of the ubiquitin ligase.

The average human cell contains approx 30,000 proteasomes, each of which contains several protein-digesting proteases. These complexes help to regulate a variety of important cellular functions, besides the cell cycle, such as the response to viral infection, the so-called stress responses, abnormal protein catabolism, neural and muscular degeneration, antigen processing, DNA repair, and cellular differentiation. At least in principle, diseases can develop if the system is either overactive or underactive. This research field is rapidly evolving, and several proteasome inhibitors are now under development (253,254).

When the cell needs to destroy a protein, it usually marks it with a chain of small polypeptides called ubiquitin. The ubiquitin–proteasome pathway degrades 90% of all abnormal, misfolded proteins and all short-lived regulatory proteins in the cell. These short-lived proteins, whose half-lives are <3 hr, account for 10–20% of all cellular proteins. As a result, proteasome inhibitors do not target individual cellular functions, but they usually affect a broad spectrum of functions. Improving the specificity of these agents remains a challenge, but, besides cancer, these agents may eventually be useful to protect tissues against ischemic injury or to maintain organs before transplantation.

The most widely used proteasome inhibitors are peptide aldehydes that potently, but reversibly, inhibit the complex's chymotrypsin-like activity, but logically they also affect a number of other intracellular proteases. The peptide boronates are more potent as well as more selective and include the reversible inhibitor pyrazylcarbonyl-Phe-Leu-boronate (PS-341) currently in phase 1 and 2 trials for patients with multiple myeloma and other malignancies. PS-341 induces apoptosis in a variety of tumor cell lines, probably as the result of effects on several regulatory pathways (including cell-cycle regulatory proteins, p53, and nuclear factor- $\kappa$ B), and exhibits potent antitumor activity in animal models, either as a single agent or in combination with conventional cytotoxics (255). In combination with geldamycin, a novel natural product that specifically binds to the heat-shock protein chaperon Hsp90, this proteasome inhibitor causes an intracellular buildup of misfolded, ubiquitinated, probably nonfunctional proteins, contributing to the antitumor activity of both agents (256).

An innovative phase 1 trial of PS-341 (Bortezomib) in patients with refractory hematologic malignancies has been published (257), with promising activity against refractory multiple myelomas (heavily pretreated) and some NHLs. The main innovation of this study is the introduction of a fairly specific pharmacodynamic test to assess the degree of inhibition of 20S proteasome activity produced *in vivo* by various doses of the test drug, the first of its class to move ahead with a filing application to the Food and Drug Administration.

Because of the rapid exit of PS-341 from the intravascular compartment, and a rather large (>500 L) volume of distribution, serum concentrations of the drug approached the limits of detection of the assay within 1 h after *iv* administration, and conventional pharmacokinetic parameters were unlikely to adequately guide dosing or area-under-the-curve (AUC) relationships with toxicities or response (258). The pharmacodynamic assay demonstrated a sigmoid PS-341 dose–20S proteasome inhibition response.

Significant inhibition of the 20S proteasome was seen within 1 h of dosing (up to 74% inhibition), after which the inhibition slowly decayed, returning toward baseline by 72 h. Toxicities at the maximum tolerated dose included thrombocytopenia, hyponatremia, hypokalemia, fatigue, and malaise, but the test drug was reasonably well tolerated at the dose of 1.04 mg/m<sup>2</sup>. Leukopenia and neutropenia were noted, but neither was dose limiting, and no episodes of febrile neutropenia were seen. Among nine assessable patients with refractory multiple myeloma, one had a complete response and eight had a reduction in paraprotein levels and/or marrow plasmacytosis.

A multicenter phase 2 study with PS-341 administered intravenously at 1.3 mg/m<sup>2</sup> to 202 patients with refractory multiple myelomas resistant to conventional chemotherapies has been reported (259). Responses were assessed using rigorous *Bladé criteria*. A complete response required 100% disappearance of the monoclonal gammopathy (M-protein), negative immunofixation testing for trace amounts of M-protein, <5% plasma cells in the bone marrow, no increase in size or number of lytic bone lesions, and disappearance of all soft-tissue tumors. Complete remissions were obtained in 4% of patients, and an additional 6% achieved a 100% reduction in M-protein but a positive immunofixation test. The overall response rate (complete and partial responses) in this heavily pretreated population of patients was an encouraging 35% (259). Twenty-one percent of patients eventually discontinued treatment due to an adverse event that included some peripheral neuropathies. A rather good median survival of 16.4 mo was reported, in this poor-prognosis group of patients.

Although these highly encouraging results with PS-341 promise rapid introduction into the clinical arena of an entirely new type of cytotoxic agent, the best and more selective anticancer drugs of the future in this setting would tag specific cyclins (e.g., cyclin D1 and cyclin E) for degradation. Recent research points toward a key role for cyclin E in breast cancer, and cyclin D1 in some NHLs and in colon cancer, acting as “de facto” potent oncogenes.

### Controlling CDK Activity by Altering Cyclin Binding to CDK

The role of cyclin D1 in circumventing the cell-cycle checkpoint in colon cancer and its relationship with intracellular catenin levels have recently come to light. In most cancers, a defect in the retinoblastoma checkpoint allows cells to divide uncontrollably and excessively. In colon cancer, this checkpoint is usually free of defect and for a long time, it was unclear how colon cancer cells achieved a proliferative advantage over normal counterparts. Several researchers linked the intracellular catenin levels with the tumor suppressor protein APC (260). The search began for the target of  $\beta$ -catenin, and a key article was published linking intracellular catenin directly to increased expression of cyclin D1, prompting cells to enter S-phase (261,262).

High levels of cyclin D1 override the checkpoint in colonic epithelial cells, generating a population of cells that would not be totally transformed, but that might acquire further mutations, such as in Ras or other oncogenes, to acquire the truly malignant phenotype. The two pathways, Ras and  $\beta$ -catenin-APC, converge into the same apparent bottleneck activating the expression of cyclin D1 (262).

Similarly, cyclin E has a pivotal role in transducing the mitogenic stimulus of several hormones, cytokines, and extracellular growth factors. Transient expression of cyclin E induces the formation and activation of CDK2 complexes, and pRb is a key substrate. It appears that pRb must be phosphorylated by CDK4/cyclin D before pRb phosphorylation by CDK2/cyclin E. Recent evidence suggests that levels of total cyclin

E (and low-molecular-weight cyclin E) in tumor tissue, as measured by Western blot assays, correlate strongly with survival in patients with breast cancer (263).

In a retrospective analysis of 395 patients with breast cancer with a median follow-up of 6.4 yr, high amounts of truncated or total cyclin E correlate with poor disease-specific survival (263,264). Among patients with early (stage I) breast cancer, all those with low levels of cyclin E were alive at 7 yr, compared with none of the patients with expression of high levels of cyclin E. If these findings are confirmed by more studies, then determination of cyclin E levels by an immunohistochemical assay might become routine in oncology units (263,264). Stimulation of the proliferation of breast cancer cells by estrogens and growth factors is accompanied by increased cyclin E and the formation of active cyclin E-cdk2 complexes.

It is obvious that selective inhibitors of cyclins E and D are good candidates for cancer therapy. Molecules designed to inhibit the binding of these cyclins to their corresponding pockets in CDK (particularly CDK2 and CDK4) are being designed and developed, and some might soon be under preclinical development. Structural studies, e.g., have focused on how RXL (cyclin-binding motif) and LXCXE (part of cyclin structure) contribute to substrate selection. RXL motif in p27 is thought to bind to a hydrophobic surface on cyclin A, which is conserved between cyclins A, B, D, and E, that lies opposite to the CDK2-binding site (265).

### **Allosteric Regulation of CDKs by Molecular Mimetics**

CKIs can provide the basic clues on how proliferation is normally controlled *in vivo*, how new classes of clinically relevant prognostic factors in cancer are established, and how molecular mimetics with high affinity for CDK and similarly potent inhibitory actions are developed.

For example, wound healing is a physiologic process in which growth of cells is stringently regulated. In a simple experimental model involving excisional wounds of full-thickness skin in rats (266), the expression of both p21<sup>cip1</sup> and p27<sup>kip1</sup> at the gene and protein levels was measured and showed an inverse gradient to that of the PCNA Ki67. Constitutive p27<sup>kip1</sup> was expressed throughout wound healing with low levels during the proliferation period of d 3 and 5, and increased levels during postmitotic and remodeling stages. By contrast, p21<sup>cip1</sup> was expressed transiently with detectable levels only between d 7 and 14, suggesting that these cell-cycle brakes may play different and coordinated roles in the normal control of cellular proliferation (266).

The detection of CKIs by immunohistochemical or other means promises to become a relevant strategy for detecting cancer risk in precancerous tissues or for better defining prognosis in malignant tissue biopsies. For example, loss of p27 protein provides independent prognostic information in breast, prostate, colon, and gastric carcinomas, and immunohistochemical staining for p27 may eventually become part of routine histopathologic processing of cancers (267). Loss of immunohistochemical staining for p21 may be prognostic in certain cancers, but conflicting results are reported in breast cancer. Reports on homozygous deletion of p16 and p15 genes suggest the value of larger, prospective studies with standardized treatment protocols to definitively establish the prognostic utility of p15/p16 deletions in acute leukemias. Larger trials and the development of a consensus on methods for deletion analysis, immunohistochemical staining, and tumor scoring will be needed to move these molecular assays from bench to bedside.

The tumor suppressor molecule p21<sup>cip1</sup> (WAF1), through its association with G1- and S-phase CDK complexes, regulates activation of pRb and E2F transcription factors. Recognition of CDK/cyclin complexes by p21 occurs in part through a protein–protein interaction with a binding groove on the cyclin subunit. The same groove has been shown to be involved in the recruitment of macromolecular CDK substrates, including pRb and E2F. Blocking of this recruitment site prevents recognition and subsequent phosphorylation of CDK substrates and offers a therapeutic approach toward restoration of p21-like tumor suppression.

Highly potent p21-derived peptide inhibitors of CDK-mediated pRb phosphorylation have been developed by several laboratories, and the molecular interactions governing cyclin-groove recognition and general rules for the development of peptidomimetic inhibitors of CDK are gradually being solved (268,269).

Another example is p16<sup>INK4A</sup>. CDK4/6 are activated on binding to cyclin D, as well as by phosphorylation at residues Thr177 in CDK6 or at Thr172 in CDK4, by CAK. INK4A represents a family of inhibitors that have CDK4 and CDK6 as target proteins. Four members of this family of inhibitors have been described, all exhibiting in common several ankyrin-like repeats: p16<sup>INK4A</sup>, p15<sup>INK4B</sup>, p18<sup>INK4C</sup>, and p19<sup>INK4D</sup> or ARF. These INK4 inhibitors can bind either to free CDK4/6 or to the cyclin D–CDK4/6 complexes, exhibiting very similar contacts. Analysis of the crystal structure of CDK6/p16 complexes by X-ray diffraction methods reveals most of the interactions between these two proteins but provides a rather static picture.

Molecular dynamics simulations (269) can provide complementary information on the nature of these interactions. Because of the large contact surface between both proteins, it is difficult to design a single, small-molecular-weight drug capable of embracing all the interactions deduced from the crystal structures and the molecular dynamic simulations. Increasing insight into the design of small molecules with sufficient selectivity to alter the T-loop conformation and distortion of the ATP-binding site is gradually being achieved. Moreover, the development of short-chain synthetic peptides capable of inhibiting normal binding of p16 to CDK4 can allow a better understanding of the key structure activity and allosteric properties of these complex molecular interactions (Fig. 14).

As discussed, gene transfer experiments, such as transfection into tumor cells in culture of active CKI genes, has enabled several laboratories to test the principle of induction of tumor regression by the activation or transfer of CKI activity. An example is that ectopic p18 expression inhibits growth and induces apoptosis of multiple myeloma cells (270). In approx 25% of human multiple myeloma cell lines, cyclin D1 is overexpressed because the *CCND1* gene is brought under the influence of immunoglobulin heavy chain (IgH) enhancer and promoter elements by the chromosomal translocation t(11;14)(q13;q32). CKI p18 predominantly binds to CDK6 and to a lesser extent to CDK4, and inactivates their kinase activity. Furthermore, p18 can efficiently block CDK6 phosphorylation by CAK/cyclin H1. Cell lines transfected with an inducible p18 expression vector not only exhibited growth inhibition, but also apoptosis (270).

Microinjection of CKI proteins has confirmed other biologic activities of CKI. Injection of the CKIs p21 and p27 in *Xenopus laevis* embryos blocked centrosome duplication (271). The centrosome nucleates the polymerization of microtubules and duplicates once per cell cycle, starting at the G1- to S-phase transition, and has a major role in organizing the poles of the mitotic spindle. Inhibition of normal cen-

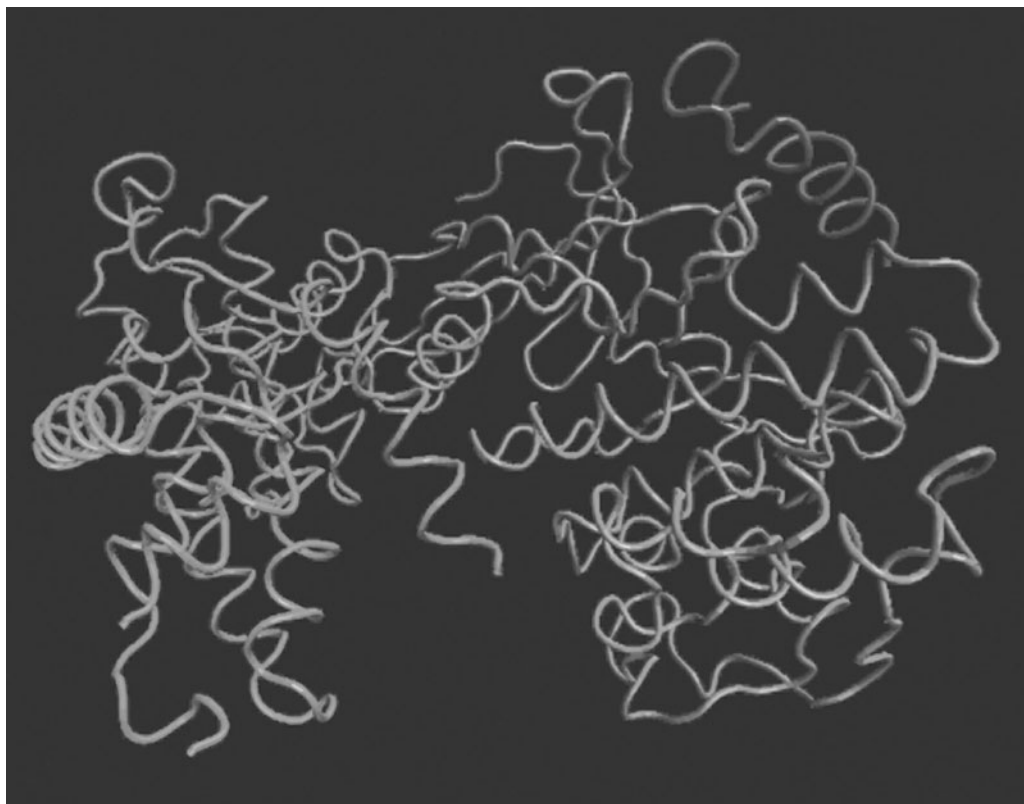


Fig. 14. Modeled structure of CDK6 (light blue) bound to 20-residue peptide (red), which corresponds to amino acids 84–103 of p16<sup>INK4</sup>, and is known to inhibit in vitro phosphorylation of pRb by CDK4–cyclin D1 (Fähræus et al. *Curr. Biol.* 1996; 6:84–91). The position of the cyclin (in green) bound to CDK6 has been deduced by superimposing C<sub>α</sub> of CDK6 residues with the equivalents in CDK6–p18<sup>INK4c</sup>–K cyclin (PDB entry 1G3N). See also Website: [www.rcsb.org/pdb](http://www.rcsb.org/pdb). (Courtesy of Oscar Villacañas and Jaime Rubio-Martínez, *Modelization of Biological Systems and Drug Design*, Research Group of the University of Barcelona.) (Color illustration in insert following page 362.)

trosome function by p21 and p27 can arrest cell growth and lead to abnormal segregation of chromosomes.

Another unexpected property of CKIs is the reported ability of p21 (WAF1/CIP1) to restore hormone responsiveness to estrogen receptor (ER)–negative breast cancer cells. Again, the overexpression of p21 with a tetracycline-inducible gene transfer vector can make ER-negative, p21–negative breast cancer cells sensitive to growth inhibition by antiestrogens, and a strong positive association has been found between the expression of p21 and the presence of ER in tumor samples from 60 patients with breast cancer (272).

In vivo protein and peptide transduction systems are being investigated by several laboratories. Recombinant proteins, such as recombinant human colony-stimulating factors, have been used in cancer patients for 15 yr (273). The inability of molecules larger than 600 Daltons to cross the plasma membrane has restricted the pharmacologic use of proteins to those that function outside the cell, such as by binding to specific cell membrane receptors. Most tumor suppressors, on the other hand, are intracellular, and

therefore cannot be administered to patients as recombinant proteins directly. Promising approaches with protein delivery tags, TAT proteins, and penetratins in particular are in progress (274), and apoptosis has been induced in neoplastic cells that overexpress E2F-1 (275). Affinity studies suggest that a dominant negative peptide (mutant of E2F-1) has a higher affinity than native E2F-1 for the main E2F/DNA-binding sites, blocking S-phase progression in some tumor cultured cell lines (276).

Some synthetic peptides can deplete the intracellular levels of several key oncoproteins that are normally stabilized by Hsp90. These oncoproteins, in the case of a promising depsipeptide called FR901228 (or FK228), include cyclin A, cyclin E, p21, p53, ErbB2, and Raf-1 (277).

### **Inhibitors of Rb Phosphorylation: The Bottleneck?**

At first sight, the Rb is the true bottleneck of most cell-cycle controlling pathways. In 1986, the tumor suppressor *Rb* gene, first identified because it was mutated in hereditary retinoblastomas, was cloned independently by three laboratories. It is a rather large gene, over 200 kb and with 27 exons. It codifies for a nuclear protein of 928 amino acids, which is constitutively expressed during the cell cycle, but with characteristic cell-cycle-dependent different degrees of phosphorylation.

Phosphorylation of pRb is done initially by D-type cyclins (in protein complexes with CDK4 or CDK6) followed later by cyclin E/CDK2 (278,279). Nearly all human tumors tested show mutations that directly or indirectly alter the normal function of Rb. Nearly all tumor-derived pRb mutants have lost the ability to repress E2F-responsive genes, and reintroduction by gene transfer of wild-type pRb into Rb<sup>-/-</sup> tumor cells leads to restoration of E2F control and cell-cycle arrest. At least six human *E2F* genes (*E2F1*–*E2F6*) and their protein products bind to specific DNA sequences as heterodimers with either DP1 or DP2 proteins. It is clear that binding to pRb converts the E2F family from transcriptional activators to potent transcriptional repressors. E2F6, unlike the other E2F family members, is an intrinsic transcriptional repressor and does not apparently interact with pRb family members. Hyperphosphorylated pRb is unable to maintain the binding and inactivation of E2F, and free E2F molecules lead to transcription of multiple genes involved in the initiation of DNA synthesis and cellular proliferation.

Using gene-expression profiling methods, it has been possible to demonstrate changes in the expression of >200 genes, most involved in cell-cycle control, by CDK phosphorylation of wild-type pRb (73). Some of these genes are involved in DNA repair or changes in chromatin structure. As expected, a significant fraction of Rb-repressed genes have promoters that are bound/regulated by E2F family members. However, targets were also identified (280) that are distinct from genes known to be stimulated by overexpression of specific E2F proteins, suggesting that some of the multiple pRb effects are not directly related to E2F proteins.

The conformation and activity of pRb is rather complex and not fully understood. It is accepted that the activity of this large tumor suppressor protein is mostly dependent on the phosphorylation status of at least 16 potential CDK phosphorylation sites. All CDK sites (Thr356, Ser807/Ser811, and Thr821) have been identified, and their phosphorylation modifies the conformation of pRb (281). The so-called m89 structural motif (identified in the m89 mutant of pRb) has enhanced growth-suppressing activity, similar to a mutant with alanine substitutions at Ser807/Ser811 (281). Moreover, the m89 region is part of a structural domain, p5, conserved antigenically and



functionally between pRb and p53. Rationally designed drugs capable of interacting with these key molecular sites may exploit the coordinated regulation of the activity of these two tumor suppressors, or at least block the conformation of the tumor suppressor pRb into its active growth inhibitory hypophosphorylated structure. This approach might prove especially valid for those tumors in which pRb is not entirely absent, e.g., as a consequence of large deletions, but retains some of the target structural motifs for either inhibition of phosphorylation or allosteric freezing into its growth inhibitory hypophosphorylated structure.

One of the seemingly tragic, and yet unexplained, features of our human genome is that the key sensors that control many of the functional interactions between Rb and p53 are linked in the same gene, making them dually vulnerable to the same genetic attack. In general, mutational events that disable the Rb pathway and facilitate cell proliferation are counterbalanced by a p53-dependent response that eliminates, or at least inhibits, incipient cancer cells. Conversely, loss of p53 function enables cells sustaining oncogenic damage to survive and proliferate. Unfortunately, the *INK4A-ARF* locus encodes in the same genetic locus two tumor suppressor proteins that regulate both the Rb and p53 pathways. ARF (p19) is a sensor of inappropriate proliferation brought about by loss of Rb (282). The product of the *p53* gene is part of the intrinsic mechanisms that monitor the cell cycle, such as inducing the expression of p21 (283,284), and mutations, deletions, or underexpression of p19<sup>ARF</sup> facilitate MDM2's degradation of p53, leaving the cell unaware of the need to initiate cell death.

To complete the picture, reactivation of telomerase activity, normally suppressed in most normal human tissues, can contribute to the carcinogenesis process (285). By contrast, despite being implicated in many important regulatory pathways, including DNA repair, protein ubiquitination, and cell-cycle control, the exact mechanisms by which inactivation of the tumor suppressor gene *BRCA1* might lead to malignant transformation, particularly in breast and ovarian cancers, remain unknown (286).

## Conclusion

CDKs play a fundamental role in the regulation of normal cell-cycle progression by controlling critical transition points. They are integral parts of checkpoint-control mechanisms that ensure proper segregation of the genetic information with high fidelity. Deregulation of CDKs has been implicated in cellular transformation and in the development of many tumor types. Understanding the enzymology and structural biology of CDKs, as well as that of their regulators, allows exploitation of these important molecules as targets for drug discovery.

Although this chapter has focused on CDKs themselves, any one of the effectors are potential drug targets also. A number of the other regulatory targets (e.g., Chk-1, Plk, CDC25) are being investigated in several laboratories. A large effort has been made in the identification of small-molecular-weight inhibitors for CDKs. Several new compounds are entering, or nearing, clinical trials. As the case with efforts on other kinases, specificity of ATP-competitive agents remains an issue. Because CDKs have so many regulators, in addition to the small-molecule approaches, different treatment modalities (e.g., gene therapy, protein therapy) remain viable and exciting possibilities.

Some cell-cycle regulatory components are implicated in the process of apoptosis. Apoptosis is frequent in tissues with high proliferative activity, such as hematopoietic cells, embryonal cells, and tumor cells. Some of the morphologic features observed in mitosis, including nuclear envelope breakdown and chromatin condensation, are

observed in apoptotic cells. Comparative gene-expression studies between populations of cells entering S-phase from quiescence and cells entering the apoptotic programs, as well as more detailed studies on the molecular interactions between the p53 gene product and INK4A-ARF, will provide better clues regarding the coordination of these two key regulatory pathways, and their relevance to cancer therapeutics (287–288).

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## Angiogenesis Switch Pathways

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**Jaume Piulats and Francesc Mitjans**

### Introduction

Few of the basic research fields within oncology have experienced such an explosive growth as the area of angiogenesis. Intense effort in the laboratories of both academia and pharmaceutical companies has led to its own translation to the clinical research. The plethora of new and old compounds shown to be either angiogenic or antiangiogenic in several laboratories in *in vitro*, *ex vivo*, and *in vivo* models has led to a parallel increase in the number of new antiangiogenic drugs entering clinical oncology trials (1). Some advanced potential drugs have stumbled in mid- or late clinical phases, however. In addition to these issues, much scientific debate has occurred, which has led to the repositioning of many antiangiogenic targets, clearly illustrating the vast activity in this field.

As basic research advances in the understanding of angiogenesis pathways, scientists learn more about the mechanism of action of both the inducers and the blockers of the tumor-induced angiogenesis. In this sense, the emerging area of translational research is leading to a better and more focused interaction between basic and clinical researchers, which will eventually lead to a better knowledge of how antiangiogenesis drugs work *in vivo*, and the definition of clinical trials that best use the potential of these new weapons in the fight against cancer.

The control of tumor neovascularization remains one of the most promising pharmacologic approaches that could be transformed into therapeutic strategies. The pioneering work of Folkman's group in the 1970s (2–4) established that solid tumors are dependent on angiogenesis, which could be the basis for cancer therapy (5,6).

Angiogenesis is the formation of new blood vessels from the existing vascular bed (7), whereas vasculogenesis is the development of vasculature from structures in the early embryo (8). Angiogenesis is a complex process primarily carried out by the extracellular matrix (ECM) and endothelial cells and is regulated by angiogenic factors: inducers and inhibitors. Physiologic angiogenesis can be found in wound healing or in endometrium vascularization during the menstrual cycle; however, the sophisticated machinery of neovascularization is an important component of many pathologic processes such as cancer, atherosclerosis, psoriasis, diabetic retinopathy, and endometriosis. This chapter concentrates on the mechanisms that direct the switch to the angiogenic

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phenotype of tumors. Knowledge of angiogenesis pathways in cancer offers two advantages: the opportunity to establish the potential prognostic relevance of tumor angiogenesis in the evaluation of cancer disease, and discovery of new pharmaceutical targets for therapy of malignant neoplasia.

The angiogenesis switch pathways balance between the positive and negative regulators of angiogenesis (9). The positive angiogenic factors include the vascular endothelial growth factor (VEGF) (also known as vascular permeability factor, VPF), which fulfills the criteria of a direct-acting angiogenesis growth factor (10), whereas the main endogenous negative regulators are angiostatin (11) and thrombospondin (12,13).

Angiostatin is an angiogenesis inhibitor produced by the primary tumor that mediates the suppression of angiogenesis in its metastases (9). This role of angiostatin has demonstrated the remote influence of the solid tumors on metastases (11). A second negative regulator is thrombospondin, which seems to be upregulated by wild-type p53 and downregulated during the switch to the angiogenic phenotype (12,14). Folkman (9) proposed that the primary tumor producing both angiogenic stimulators and inhibitors could direct the evolution of the tumor depending on the amount of these mediators in the blood.

This apparent simplicity masks more complex processes in which many additional factors are involved. We wish to emphasize the role of vitronectin receptor (integrin  $\alpha v \beta 3$ ) and proteolytic enzymes (e.g., metalloproteinases) in defining the angiogenesis pathways, owing to their pivotal role in the design of new therapeutic strategies (15).

The clinical trials done to date with many compounds have not shown significant effects on tumor regression (16). Nevertheless, new inhibitors are being developed. A more immediate clinical application is the assessment of tumor angiogenesis as a prognostic marker in malignant neoplasia.

## Mechanisms of Tumor Neovascularization

A tumor is unable to grow larger than approx 1 mm<sup>3</sup> without developing a new blood supply. Neovascularization is thus controlled by tumor cells, which may secrete angiogenic factors to attract endothelial cells. The activated endothelial cells, in turn, may produce paracrine growth factors for the tumor. This cross talk between tumor and endothelial cells is one of the major features in angiogenesis. A second feature is the delicate equilibrium between the endogenous inducers and inhibitors of neovascularization (Fig. 1). Normal cells secrete low levels of inducers and high levels of inhibitors; however, when progressing to malignancy, the tumor cells tip this balance to an angiogenic phenotype.

## Tumor Angiogenic Switch

The essential role of angiogenesis in tumor progression and metastasis and the balance between positive and negative regulatory factors led to the idea of an angiogenic switch that is activated in tumor angiogenesis. Cells may switch to an angiogenic phenotype during progression toward tumorigenicity, and this switch often takes place early, before tumorigenicity. In vivo switches develop angiogenesis in a graded fashion through several stages. In melanoma, for example, a significant increase in vessel counts is first observed in the progression from benign to dysplastic nevi. A further increase in vessel counts from radial to vertical melanoma has been correlated with greater risk of recurrence, metastasis, and death. Similarly, angiogenesis in breast car-



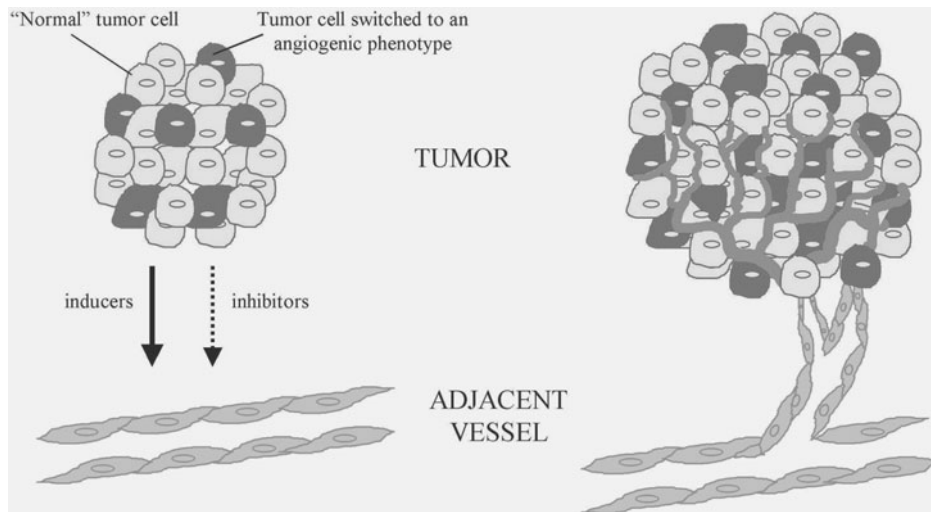


Fig. 1. Angiogenic switch. Some cells in the developing tumor switch to an angiogenic phenotype. The angiogenic cascade starts with the initiation phase in which angiogenic factors are released by tumor and accessory cells. The balance between the endogenous inducers and inhibitors is a key feature in angiogenesis. Endothelial cells, in response to angiogenic factors, proliferate and invade the stroma toward the tumor mass. During this step, integrins and proteolytic enzymes play a capital role. Later phases lead to differentiation of the newly formed capillaries to mature vessels. The cross talk between tumor and endothelial cells is a second key feature in angiogenesis.

cinoma is first noted in ductal carcinomas *in situ* (DCIS). In addition, transgenic mouse models have allowed researchers to study and define the angiogenic switch in early stages of tumor development preceding the appearance of solid tumors (17,18). These findings suggest that activation of angiogenesis—the switching on—is a discrete event in tumor development.

Potential switches can be any genetic alteration affecting oncogenes or tumor suppressor genes (TSGs), which may select for tumor cell clones with not only enhanced proliferation and survival potential but also with increased angiogenic growth factor production. In fact, one of the common features of a growing tumor mass appears because of the oxygen and nutrient consumption, which leads to a tumor microenvironment characterized by low oxygen tension. Hypoxia-inducible factors (HIFs) are activated in response to hypoxia. HIF-1 is a transcriptional activator that functions as a master regulator of oxygen homeostasis (19). Once overexpressed, HIF signals through an expression increase of several proteins, many of them involved in tumor angiogenesis (19). Nevertheless, hypoxia is not the only potential switch because HIF itself is activated under normoxic conditions (20), by cytokines (21) or even by the overexpression of the antiapoptotic bcl-2 protein (22).

It is clear that changes in the balance between positive and negative signals mediate the angiogenic switch. A net balance of inhibitors over activators would maintain the switch in the off position, whereas a shift to an excess of activating stimuli would turn angiogenesis on.

### **Endothelial Cells: Key Component in Angiogenesis**

Most tumor vessels sprout from preexisting ones and are thus derived from normal, nonmalignant host cells. Although they are composed of normal cells, vessels elicited by tumors are frequently distinct from those in adjacent normal tissue: these vessels are leaky and abnormal in size and shape. Endothelial cells appear to be fenestrated and they increase cell adhesion molecules (CAMs) such as E-selectin (23) and specific integrins such as  $\alpha v\beta 3$  (24) that are essential for their viability during growth. Microcapillary endothelial cells from different organs exhibit a differential display of cell receptors and may be able to be targeted by specific peptide sequences (25). Activated endothelial cells release a variety of growth factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF), which can maintain endothelial cell activation as well as act as paracrine stimulators of tumor cells. They are also capable of producing a variety of factors that may inhibit tumor cell growth, such as interleukin-6 (IL-6), to which early stage melanomas have been shown to be sensitive but to which late-stage melanomas are often resistant (26,27).

Angiogenesis generally occurs in at least three steps: induction–initiation, proliferation–invasion, and maturation–remodeling (Fig. 1). In the first stage, angiogenic inducers, such as growth factors or cytokines, are released both by the tumor cells themselves and by the accessory cells recruited to the site. These factors stimulate vascular cell proliferation and invasion, thereby promoting blood vessel growth toward the tumor mass. One important result associated with cell invasion is that changes in the CAMs enable endothelial cells to interact with surrounding stroma wherever the endothelial cell is proliferating and invading. In turn, the adhesion molecule–mediated signaling ensures continued cell survival, proliferation, and invasion. Later phases of angiogenesis involve a halt in proliferation and cell differentiation and both tubular structure and lumen formation leading to blood circulation. The basal lamina is modified and the newly formed blood vessel is surrounded by differentiated pericytes and smooth muscle cells (28).

### **Vasculogenic Mimicry**

Angiogenesis may not be the only mechanism by which tumors acquire a microcirculation. Sood et al. (29) showed that the generation of microvascular channels by tumor cells is a process termed *vasculogenic mimicry* that does not imply the participation of endothelial cells. This phenomenon describes the embryonic-like ability of aggressive tumor cells to form vascular networks. Vascular endothelial cadherin (VE-cadherin) is exclusively expressed by highly aggressive melanoma cells, and its downregulation expression abrogates their ability to form those networks. These results suggested that VE-cadherin is critical in melanoma vasculogenic mimicry (30). Moreover, Shirakawa et al. (31) used a model of inflammatory breast cancer xenograft (WIBC-9) to show that these tumors exhibit vasculogenic mimicry without endothelial cells revealing expression of human Flt-1 and human Tie-2 and the absence of human CD-31 and human thrombin receptor.

The presence of tumor cell–lined vascular channels has been known for years, but its significance is still not known. MacDonald et al. (32) suggested that cancer cells contribute in only a small proportion of the lining of blood vessels in tumors. According to these investigators, morphometric studies using immunohistochemistry (IHC) and green fluorescent protein–transfected tumor cells suggest that human colon cancer cells constitute only 3% of the vessel surface in tumors grown orthotopically in mice.

Some investigators call this phenomenon mosaic vessels (33). Therefore, further knowledge is needed to understand the value of antiangiogenic therapy on tumors that express this vasculogenic mimicry switch.

Much more recently Rafii et al. (34) revealed the important contribution of circulating bone marrow–derived endothelial progenitor cells in tumor neovessel formation. This fact illustrates again the complexity of the tumor neovascularization process and gives additional clues to the angiogenic switch mechanisms.

### ***Inducers of Angiogenesis***

Several in vitro and in vivo bioassays have been developed to mimic the complex process of angiogenesis; two in vitro assays are particularly important and use either endothelial cell proliferation or endothelial cell migration. Both assays are often complemented by the use of an in vivo assay, such as implants into the normally avascular cornea of rabbits or rodents (i.e., corneal pocket assay). Using these assays, a number of inducers of angiogenesis have been identified. Most of the angiogenesis inducers are part of a complex system that involves soluble ligands and endothelial cell receptors, as in the cases of the VEGF/VEGF receptor (VEGFR) and the FGF/FGF receptor (FGFR) systems.

#### ***Vascular Endothelial Growth Factor***

VEGF is one of the primary angiogenic factors because it is the first factor produced during embryogenesis to control both vasculogenesis and angiogenesis (35). Moreover, it is the only growth factor described to date whose null mice are not viable (36,37). VEGF was first identified by its ability to elicit vascular permeability; subsequently, this factor was shown to be a mitogen for endothelial cells, and it has been described as a potent inducer of angiogenesis in vivo (38). Three related endothelial growth factors—VEGF-B, VEGF-C, and VEGF-D—have been identified (39–41), and VEGF-C and VEGF-D have been described as the specific inducers of lymphangiogenesis. VEGF is induced by hypoxia and hypoglycemia and it binds to three specific receptors of the tyrosine kinase family (flk, flt-1, and flt-4), which may be upregulated on tumor endothelial cells (42). Additionally, the VEGF/VEGFR system is highly specific; although VEGF may be expressed by a number of cells, its receptors are expressed primarily by endothelial cells. VEGF may be stored in the ECM as a heparin-binding protein bound to heparan sulfate proteoglycans. When angiogenesis is required, VEGF is released from the ECM (43) or it is newly produced; its expression is often upregulated in many tumor cells. Some oncogenes, such as mutated *ras*, transcriptionally activate expression of VEGF (44).

#### ***Fibroblast Growth Factors***

Composed of at least nine forms, FGFs constitute a family of growth factors characterized by high-affinity binding to heparin; bFGF and acidic FGF (aFGF) are the forms that have been most widely studied. FGFs are unusual in that they lack the signal sequence for secretion; however, both may be released from cells under specific conditions. Both aFGF and bFGF bind receptors on endothelial cells that are transmembrane tyrosine kinases and are thus coupled through the signal transduction cascade. At least four FGFRs (FGFR1–4) exist and are widely expressed (45). Like receptors, FGFs are expressed in a number of tissues including tumors and endothelial cells (46). FGFs have an extremely strong affinity for heparin and are sequestered in

the ECM until proteolytic enzymes degrade ECM during angiogenesis. FGF is a potent mitogen and chemotactic factor for endothelial cells. It also induces formation of capillary-like structures (47) and has shown angiogenic activities *in vivo*.

### *Angiopoietins and Tie-2 Receptors*

At least six isoforms of angiopoietins (Ang) are described in the scientific literature that potentially could be involved in the neovascularization of tumors. While Ang-1 and Ang-3 are agonists of the Tie-2R, Ang-2 and Ang-4 are described as antagonists of the Tie-2R (48). Ang-2 may have an agonistic effect at high concentrations (49). Although the physiologic relevance of this finding still remains to be defined, it clearly illustrates the complexity of this emerging system in angiogenesis. To further increase this complexity, some reports describe opposite effects of a given agonist. Although Ang-1 promotes tumor angiogenesis *in vivo* (50), it was reported that Ang-1 can inhibit tumor growth and ascites formation in a murine model of peritoneal carcinomatosis (51). Moreover, the Tie-2 homolog, Tie-1R, is considered an orphan receptor because its ligand has not been elucidated (52). In addition, some researchers have found so-called Ang-like molecules with homology to the Ang, although their receptors are not well defined (53).

Like VEGFR, Tie-2R is highly tissue specific because it is primarily expressed in endothelial cells. On the other hand, Ang are mainly secreted by mesenchymal cells, especially pericytes (54), that participate in the differentiation of endothelial cells under a given stimulus. Although Tie-2R's role in tumor angiogenesis is under intense investigation, it is important to note the significant tumor growth inhibition achieved in mouse models by means of adenoviral delivery of a recombinant soluble Tie-2R (55).

### *Ephrin and Eph Receptors*

The Eph receptor tyrosine kinase (RTK) family represents a new class of RTK, and its role in angiogenesis is beginning to emerge. Unlike other families of RTK, which bind to soluble ligands, EphRs interact with cell surface-bound ephrin ligands, activating signaling pathways in a bidirectional fashion, through both the EphR and ephrin ligands (56). This system is more complex than others because it has 14 receptors and 8 ligands (57,58). EphR and ephrin ligands have a critical role in vascular development during embryogenesis, although the function of these molecules in pathologic angiogenesis is not well characterized. Preliminary data, however, suggest a role in promoting angiogenesis in tumors: soluble EphA2R inhibited tumor neovascularization in a dorsal vascular window assay (59).

### *Transforming Growth Factor- $\beta$*

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a homodimeric polypeptide secreted in a biologically inactive, latent form. This form may be activated *in vitro* by heat, acidification, and proteases (60), thus providing a regulatory mechanism. TGF- $\beta$ , similarly to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), affects endothelial cells in a dual manner. They inhibit endothelial cells *in vitro* but stimulate angiogenesis *in vivo* (61). It has been proposed that TGF- $\beta$  induces angiogenesis by an indirect mechanism: it is highly chemotactic for monocytes and other accessory cells that, in turn, release angiogenic factors that are mitogenic for endothelial cells (62). TGF- $\beta$  and its receptors are expressed in many tissues, and the differences in the response to TGF- $\beta$  are attributable to differences in the surface expression of TGF receptor.

### *Tumor Necrosis Factor- $\alpha$*

TNF- $\alpha$ , a secreted protein synthesized primarily by activated macrophages and by some tumor cells (63), was first described as causing solid tumor necrosis and regression. TNF- $\alpha$ , like TGF- $\beta$ , has paradoxical angiogenic activity. In vitro, TNF- $\alpha$  has an antiproliferative effect on endothelial cells, while in vivo it induces angiogenesis. The angiogenic activity in vivo is, in turn, also dual. When used at low concentrations, TNF- $\alpha$  induces angiogenesis—both vessel growth and endothelial cell proliferation. At high concentrations, TNF- $\alpha$  inhibits angiogenesis. Some investigators (64) suggest that the mode of delivery of TNF- $\alpha$  to endothelial cells may have a role in their response.

### *Platelet-Derived Endothelial Cell Growth Factor*

Platelet-derived endothelial cell growth factor (PD-ECGF) was first described in platelets as a new angiogenic factor; however, PD-ECGF is not a mitogen for endothelial cells, so the name is inappropriate (65). When cloned and sequenced, the gene for human thymidine phosphorylase matched that of PD-ECGF. Consequently, many investigators have described thymidine phosphorylase as an angiogenic enzyme. It is now known that the angiogenic molecule is not the enzyme by itself but, rather, the product of thymidine phosphorylase action on thymidine: 2-deoxy-D-ribose is mainly responsible for the angiogenic activity (65). PD-ECGF/thymidine phosphorylase is a particularly intriguing molecule to study. It is an angiogenic enzyme and not a classic growth factor, and its expression is exceptionally high in most solid tumors compared with normal tissues.

### *TGF- $\beta$ and Epidermal Growth Factor*

TGF- $\beta$  and epidermal growth factor (EGF) share 40% homology, and both bind to EGF receptor. TGF- $\beta$  is expressed in macrophages and some tumor cells and, like EGF, stimulates the proliferation of endothelial cells in vitro. Both factors induce migration in vitro and capillary-like tube formation and angiogenesis in vivo (66), although EGF is less potent.

### *Other Angiogenic Compounds*

A number of other angiogenic molecules have been described, but in most cases, the mechanism of action either is not completely known or understood or appears to be indirect. Angiogenin, e.g., a protein of the pancreatic ribonuclease family, is angiogenic in vivo but not in vitro (67). The ILs have a role in controlling angiogenesis, either inducing it like one of the following—IL-8 that has been shown to potently stimulate angiogenesis (68), IL-1 $\alpha$  that promotes angiogenesis through the upregulation of VEGF (69), IL-17 (70), or IL-18 (71)—or by inhibiting angiogenesis like IL-4 (72) and IL-12 (73). Even osteopontin (74), thrombin (75), some prostaglandins (76), and nicotine (77) have been reported to have angiogenic activity. A biologic fragment of human tyrosyl-tRNA synthetase also induces angiogenesis in vitro and in vivo (78). This finding has an interesting parallelism with the many antiangiogenic biologic fragments from inactive natural whole proteins.

### ***CAMs and Angiogenesis***

Cell adhesion receptors mediate processes of cell adhesion, proliferation, migration, and invasion involved in the cascade of angiogenesis. Angiogenesis not only depends on growth factors, but is also influenced by CAMs. This fact is illustrated by

the experimental studies in which both tumor and endothelial cells are subcutaneously injected into nude animals (79). The tumors formed after injection of both cell types were profoundly vascularized throughout by the tubular structures formed by the injected endothelial cells. Moreover, the tumor mass was an average 5.8-fold as large as control tumors that were grown without exogenous endothelial cells (79). In addition, treatment of tumors thus formed after injection of both types of cells with antagonists of CAMs involved in tumor–endothelial cell interaction could downmodulate tumor growth to the same level as that of tumors formed from tumor cell injection alone (79). Similar results were obtained using a different experimental approach (80).

Thus, the interaction of tumor cells and endothelial cells in orderly tumor angiogenesis is highly dependent on the action of CAMs to mediate the adhesion of cancer cells to endothelial cells, the inhibition of which retards tumor growth and angiogenesis.

At least four families of cell adhesion receptors can be classified based on their biochemical and structural characteristics: the selectins, the immunoglobulin (Ig) supergene family, the cadherins, and the integrins. Members of selectins, transmembrane receptors that mediate interaction to sialylated glycans, include P-selectin, L-selectin, and E-selectin. Both P- and E-selectin may also be expressed in a soluble form. P- and E-selectin are upregulated in endothelial cells after exposure to inflammatory agents such as TNF- $\alpha$ , lipopolysaccharide, and IL-1b (23). E-selectin is expressed in proliferating endothelial cells of the childhood hemangiomas (a benign tumor composed of endothelial cells). Supporting a possible functional role for E-selectin in angiogenesis, Koch et al. (81) have demonstrated that soluble E-selectin, although unable to induce endothelial cell proliferation in vitro, stimulated endothelial cell migration in vitro and angiogenesis in vivo. Nevertheless, E-selectin and P-selectin knockout (KO) mice showed no defects in blood vessel formation (82).

Further studies implicate members of the Ig supergene family in angiogenic processes. These CAMs share the characteristic repetitive extracellular Ig-like domains and mediate heterophilic cell–cell adhesion (83). Members of the family include intercellular adhesion molecule-1 (ICAM-1), ICAM-2, ICAM-3, vascular cell adhesion molecule-1 (VCAM-1), and platelet/endothelial cell adhesion molecule (PECAM). Similarly to selectins, VCAM-1 and ICAM-1 can be expressed as soluble forms. Whereas ICAM-2 and PECAM are highly expressed in both resting and activated endothelial cells, ICAM-1 and VCAM-1 are upregulated after stimulation with inflammatory cytokines such as IL-1, TNF- $\alpha$ , and interferon- $\gamma$  (IFN- $\gamma$ ). ICAM-3 is highly expressed in tumor endothelial cells but not in sites of inflammation (84). Only one member of Ig superfamily is clearly involved in angiogenesis. Soluble VCAM-1 induces endothelial cell migration in vitro and angiogenesis in vivo (81) although, like soluble E-selectin, it is unable to induce endothelial cell proliferation.

The cadherin family of cell–cell adhesion molecules, composed of E-cadherin, P-cadherin, L-cadherin, and VE-cadherin, are transmembrane proteins that mediate homophilic cell–cell adhesion in a calcium-dependent manner. Antibodies (Abs) against VE-cadherin inhibit tumor angiogenesis and tumor growth without affecting vascular permeability (85,86). In addition to VE-cadherins, other members of this family may have a role in different stages of angiogenesis (87). The loss of cadherins, e.g., may promote increased invasion of activated endothelial cells, as seen in invasive tumor cells.

Integrins are heterodimeric transmembrane cell–ECM adhesion receptors composed of a  $\beta$ -chain noncovalently associated with an  $\alpha$ -chain. At least 15  $\alpha$ -subunits and 8  $\beta$ -

subunits have been identified, which can combine to give at least 20 integrins. This combination, in turn, defines their cellular and adhesive specificity. Integrins predominantly mediate cell–ECM interactions, although some members may intervene in cell–cell adhesive events. Ligands for integrins include fibronectin, collagen, laminin, vitronectin, thrombospondin, and fibrinogen. Several integrins recognize the tripeptide sequence of Arg-Gly-Asp within the ligands. In recent years, a growing body of evidence has suggested a critical role for integrin receptors in the regulation of angiogenesis and vascular development. For example, ECM molecules that are ligands for integrins are abundant in the surrounding vascular matrix and subendothelial basement membrane of blood vessels. This situation leads to inevitable changes in the integrin repertoire of new vessels, thus providing evidence of the importance of integrins in angiogenesis. Endothelial cells express members of the  $\beta 1$ ,  $\beta 3$ , and  $\beta 5$  subfamilies, and stimulation of these cells with bFGF *in vitro* causes increased expression of  $\beta 1$  and  $\beta 3$  integrins. *In vitro* experiments have shown the involvement of  $\alpha 6\beta 1$  (laminin receptor) in endothelial cord formation but not in capillary lumen formation, which seems to require the participation of  $\alpha 2\beta 1$  integrin (88).  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins have been involved in angiogenesis through upregulation promoted by VEGF (89). Notwithstanding, integrin function during blood vessel formation *in vivo* has been studied most extensively for  $\alpha v\beta 3$ .

The integrin  $\alpha v\beta 3$ , also named the vitronectin receptor (VNR), is minimally expressed in quiescent blood vessels, but it is highly upregulated after stimulation by either angiogenic growth factors or tumors (24).  $\alpha v$  integrins are highly expressed on the tips of sprouting angiogenic blood vessels (90). *In vitro*, both Abs against  $\alpha v\beta 3$ , and  $\alpha v\beta 3$  and synthetic Arg-Gly-Asp-containing peptides, affected microvessel outgrowth from rat aorta rings embedded in fibrin gels (91–93) and endothelial cord formation (94,95). Furthermore, Abs and cyclic Arg-Gly-Asp peptides used *in vivo* as antagonists of  $\alpha v\beta 3$  blocked angiogenesis induced by cytokines and solid human tumors in several models such as the chick chorioallantoic membrane (96), rabbit cornea (97), mouse retina (98), nude mouse (99) and human skin–severe combined immunodeficiency (SCID) mouse chimeras (100).

### **Role of VNR in Angiogenesis**

Integrin  $\alpha v\beta 3$ , the VNR, considered the most promiscuous member of the integrin family, recognizes many ligands: vitronectin, fibronectin, fibrinogen, laminin, collagen, von Willebrand's factor, osteopontin, thrombospondin, tenascin, adenovirus penton base, bone sialoprotein, matrix metalloproteinase-2 (MMP-2), and other Arg-Gly-Asp-containing proteins. This feature confers to any  $\alpha v\beta 3$ -expressing cell the ability to adhere to, migrate, and respond to almost any environment it may encounter (101). VNR has low expression in normal tissues but is upregulated in activated endothelial cells. In addition,  $\alpha v\beta 3$  is expressed in some invasive tumors such as late-stage glioblastomas (102) and malignant melanomas (103). Interestingly, overexpression is well correlated with the degree of malignancy and invasion in melanomas. Although normal melanocytes, nevi, and noninvasive radial growth phase melanomas are negative for its expression, both invasive vertical growth phase and metastatic melanomas are highly positive (104). This differential expression has been proposed as a prognostic factor (105). The use of the integrin has been studied as a therapeutic target in melanoma lesions (106–111) (Fig. 2).

The highly restricted expression and upregulation during neovascularization suggests that  $\alpha v\beta 3$  may have a functional role in angiogenesis. Antagonists of  $\alpha v\beta 3$  (both

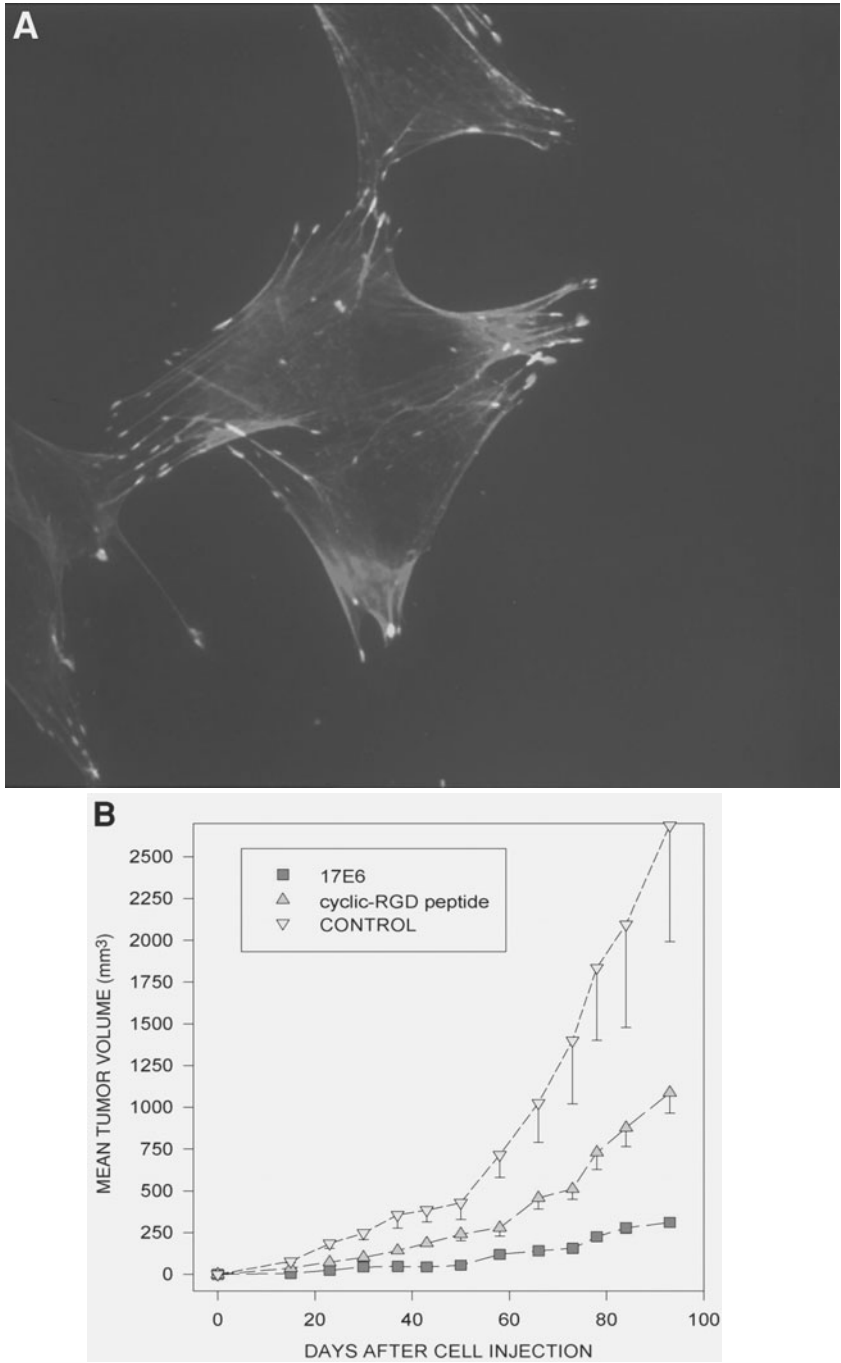


Fig. 2. (A) Confocal image of human melanoma cells stained with the anti- $\alpha_v$  integrin MAb 17E6. Secondary antibody is fluorescein isothiocyanate labeled and actin is visualized using Phalloidine-TRITC. Note the focal contact staining with 17E6. For further information, see text and refs. 106 and 107. (Color illustration in insert following page 362.) (B) M21 human melanoma cells were subcutaneously injected into nude mice. Therapeutic drugs were MAb 17E6 and cyclic-RGD peptide. MAb 17E6 was administered at d 0, 32, 39, 46, and 53. Peptide was injected daily. For further details see text, and refs. 106 and 107.



monoclonal antibodies (Mabs) and cyclic Arg-Gly-Asp-containing peptides) prevented blood vessel formation in a number of *in vitro* and *in vivo* models of angiogenesis (24,95–111). A related integrin,  $\alpha\beta 5$ , is involved in angiogenesis. In an elegant study using the rabbit corneal model, Friedlander antagonists of  $\alpha\beta 3$  integrin inhibited angiogenesis induced by bFGF but had little, if any, effect on VEGF-induced angiogenesis. By contrast, antagonists of  $\alpha\beta 5$  integrin were able to block VEGF, but not bFGF-induced angiogenesis. Most important, antagonists of  $\alpha\text{v}$  integrins inhibited both cytokine- and tumor-induced angiogenesis. These findings define two distinct pathways leading to angiogenesis depending on the particular  $\alpha\text{v}$  integrin involved. Studies have elucidated the possible mechanisms by which  $\alpha\beta 3$  antagonists inhibit angiogenesis. Both MAb and cyclic Arg-Gly-Asp peptides selectively induce programmed cell death (apoptosis) in angiogenically activated endothelial cells *in vivo*.  $\alpha\beta 3$  provides survival signals when it interacts with denatured collagen (112). Subsequent studies showed that systemic administration of  $\alpha\beta 3$  antagonists promotes apoptosis in developing but not in resting blood vessels (109). The results support the hypothesis of a key role of the  $\alpha\beta 3$  integrin in angiogenesis.

At least two controversial situations show the need for further investigations in this field. First, patients with Glanzmann thrombasthenia lack the  $\beta 3$ -subunit and are defective for  $\alpha\beta 3$  integrin. Although they have normal blood vessels, their wound-repair function is severely compromised (113). Such patients might develop blood vessels by an alternative mechanism involving  $\alpha\beta 5$  integrin. Second, some  $\alpha\text{v}$  KO mice not only survive a short time after birth, but also have normal blood vessels in most organs, such as the lung (114). Nevertheless, these animals develop cerebral hemorrhage, thus suggesting abnormalities in brain blood vessel development. The fact that vessels of tissues of mesodermal origin (i.e., lungs) are formed through vasculogenesis (which does not depend on  $\alpha\beta 3$ ) while vessels of tissues of endodermal origin (brain) are formed through angiogenesis ( $\alpha\beta 3$  dependent) can explain this differential behavior (27).

Both  $\beta 3$  and  $\beta 3$ - $\beta 5$  double KOs have increased angiogenesis and increased tumor growth rate (115). This finding indicates that in KO mice,  $\alpha\beta 5$  integrin does not compensate for the lack of  $\alpha\beta 3$  in promoting angiogenesis. These results were obtained, however, in animals that lack  $\alpha\beta 3/\beta 5$  integrins from early in embryogenesis. A definitive help in understanding this issue could come from the use of conditional KO mice, in which  $\alpha\text{v}$  integrins would be present until the formation of adult mice and could be knocked down during a given time frame to study the role in tumor angiogenesis. Meanwhile, some researchers have hypothesized that integrins could be negative regulators of angiogenesis and that the drugs targeting them may be acting as agonists rather than antagonists (116). Other investigators indicate that  $\alpha\beta 3$  can provide a feedback mechanism, acting as a biosensor to facilitate integrin-mediated death when endothelial cells engage an inappropriate ECM (117). Thus, unligated integrins can act as negative regulators of cell survival, initiating a process referred to as “integrin-mediated death.”

Experimental evidence increases for a supporting role for  $\alpha\text{v}$  integrins, especially  $\alpha\beta 3$ , in the control of tumor angiogenesis. Research suggests that blockade of this integrin using different approaches serves to block tumor-induced angiogenesis and tumor growth. Several snake venom disintegrins, e.g., inhibit angiogenesis and tumor growth by a selective  $\alpha\beta 3$  blockade of endothelial cells (118). Other compounds, antagonists of  $\alpha\beta 3$ , such as Cilengitide (a cyclic Arg-Gly-Asp peptide) (119) and synthetic peptidomimetics, are also potent inhibitors of angiogenesis and tumor growth (120). Notable as well are the ECM-derived peptides (fragments of natural, not

angiogenically active molecules) that inhibit  $\alpha v\beta 3$  and both tumor angiogenesis and tumor growth (121). Examples are tumstatin (122), canstatin (123), arresten (121), angiostatin (124), and endostatin (125). An additional and certainly important issue concerns the fact that both nonsteroidal anti-inflammatory drug inhibitors of Cox2 (126), and clinical therapies using TNF and IFN- $\gamma$  (127), are associated with the suppression of integrin  $\alpha v\beta 3$  function and signaling in endothelial cells (128). Finally, the  $\alpha v\beta 3$  integrin has been used for tumor targeting (129) and tumor (angiogenesis) imaging (130).

### **Proteases**

Angiogenesis is not only regulated by the action of growth factors and CAMs but also is influenced by many other molecules. Among them, enzymes that degrade the ECM provide a suitable environment for endothelial cell migration through the adjacent stroma. At least three families of proteolytic enzymes could play a role in angiogenesis and tumor progression: the Ser proteases (including urokinase plasminogen activator [uPA]), the MMPs, and the Cys proteases (cathepsins B and L) (131). Expression of uPA receptor (uPAR) on endothelial cells, for example, is increased by tumor cell-conditioned medium (132) and VEGF (133). In addition, uPAR in conjunction with integrins (134) could direct proteolysis at the leading edge of migrating endothelial cells; uPAR upregulation is a pivotal feature in angiogenic processes. Antagonists of uPAR showed antiangiogenic activity both in vitro and in vivo (135), although uPA KO mice have normal angiogenesis (36). uPA may be indirectly involved in angiogenesis regulation. Recent studies showed that a fragment of plasminogen acts as an endogenous inhibitor of angiogenesis (11). Other groups suggested that uPA (136), macrophage metalloelastase (137), pancreas elastase, or metalloproteinases (138) could be enzymes responsible for the generation of angiostatin.

MMPs form a family of zinc-dependent endopeptidases with a broad spectrum of activity that is secreted as inactive zymogens (131). The MMPs may be classified as collagenases, gelatinases, and stromelysins, depending on their substrate specificity. MMP overexpression may be detected in tumor tissue or in adjacent stroma but not in surrounding normal tissue (139,140). Similarly, in vitro MMP overexpression in endothelial cells was described after bFGF or TNF- $\alpha$  stimulation (141). Both natural inhibitors, known as tissue inhibitors of MMP (TIMPs), and synthetic inhibitors of MMPs have been described as potential antiangiogenic and antimetastatic drugs. TIMPs are active in blocking lung colonization by tumor cells and angiogenesis in vivo. The MMP inhibitor Batimastat inhibits angiogenesis (142) and tumor growth (143) in experimental models. MMP-2 has been colocalized with  $\alpha v\beta 3$  in activated endothelial cells in vivo (144), supporting the importance of both molecules in angiogenesis. A new family of MMPs whose members exhibit a transmembrane domain has been discovered. These membrane-type MMPs are able to bind inactive gelatinases, such as MMP-2, mediating its activation and focusing the proteolytic activity (145).

### **Lymphangiogenesis**

The study of tumor angiogenesis should consider an additional mechanism that has an important role in the spread of cancer cells within the body—the process of lymphangiogenesis. Several groups have shown that VEGF-C and VEGF-D are regulators of lymph vessel growth and enhancers of lymphatic metastasis (146,147). These growth factors appear to be the ligands of VEGFR-3, which was found to be predominantly

expressed on lymphatic vessels during development (148). Further investigation (148) suggests that VEGFR-3 and its ligands VEGF-C and -D may have a crucial role in tumor-induced lymphangiogenesis.

These data indicate that angiogenesis and lymphangiogenesis are induced and controlled by different members of the VEGF family. Thereby, VEGF-A and -B and RTKs VEGFR-1 and -2 mediate both vasculogenesis and angiogenesis, whereas VEGFR-3 and VEGF-C and -D modulate lymphangiogenesis. These findings suggest new approaches for limiting the spread of lymphatic metastasis by means of inhibiting the VEGFR-3 function.

### **Endogenous Inhibitors of Angiogenesis**

A variety of molecules produced by normal mammalian cells can inhibit angiogenesis although they represent approx 25% of all known inhibitors. Most inhibitors produced by mammalian cells are effective in the form in which they are secreted (e.g., thrombospondin), but some are proteolytic products of the extracellular cleavage of molecules that are angiogenically inactive when intact (e.g., angiostatin [11], endostatin [149], tumstatin [122], arresten [121], and vasostatin [150]). Of the approx 200 compounds with antiangiogenic activity described to date, only the most representative are reviewed here. Additional information can be obtained in recent reviews (151–154).

Thrombospondin, an ECM component, is less abundant in human breast cell lines than in normal cells, or in immortal rat tracheal epithelial cells compared with primary cells. Thrombospondin is downregulated when normal human fibroblasts immortalize as a result of loss of p53. The decrease in thrombospondin shifts the phenotype of the cells from antiangiogenic to angiogenic (155). Because thrombospondin can also inhibit *in vivo* angiogenesis, it may be considered as an angiogenesis inhibitor.

Two new endogenous angiogenesis inhibitors have been discovered: angiostatin and endostatin. They belong to a new family of antiangiogenic agents produced from the cleavage of natural nonangiogenic molecules, which rapidly increases the number of members. The first to be described was angiostatin (11). It derives from plasminogen, and only the fragment, not the whole plasminogen, has an antiproliferative effect on endothelial cells *in vitro* and blockade of neovascularization *in vivo*, as well as prevents the growth of primary tumor and metastases (11,156). Angiostatin has no detectable direct effect on tumor cells. The mechanism by which the primary tumor produces angiostatin from plasminogen has been investigated (136–138). The exact mechanism of action of angiostatin is not clear (157,158), although several investigators report binding to a given receptor such as adenosine triphosphate synthase on the surface of human endothelial cells (159) or  $\alpha v \beta 3$  integrin (124). It appears that angiostatin does not selectively act in endothelial cells because it also binds smooth muscle cells, blocking their proliferation and migration *in vitro* (160). Angiostatin, like many other antiangiogenic compounds, has been used *in vivo* in antiangiogenic gene therapy experiments (161).

Endostatin is a proteolytic fragment of collagen XVIII (149). Endostatin has activity similar to that of angiostatin; it may block endothelial proliferation *in vitro* and *in vivo* in both angiogenesis and primary tumor growth (162), without significant direct activity against tumor cells. Endostatin has been used *in vivo* in antiangiogenic gene therapy experiments (161,163). Concerning its mechanism of action, many receptors have been described, such as VEGFR (164); integrins such as  $\alpha 2 \beta 1$  (165),  $\alpha 5 \beta 1$ , or  $\alpha v \beta 3$  (125); and cell-surface glypicans (166).

Other members of this family of fragments coming from natural, not angiogenically active, molecules with proven antiangiogenic activity are tumstatin, a fragment of  $\alpha 3$  type IV collagen (122); canstatin, a fragment of the  $\alpha 2$  chain of type IV collagen (123); arresten, a fragment of the  $\alpha 1$  chain of type IV collagen (121); and PEX, a noncatalytic fragment of MMP2 (167). Other investigators have described both pro- and antiangiogenic peptides coming from fragments of two aminoacyl-tRNA synthetases (168). In addition, the recently recognized, but steadily growing, knowledge of the relationship between the coagulation and angiogenesis pathways has research and clinical implications (169). Cryptic domains can be released from hemostatic proteins through proteolytic cleavage, which may act systemically as angiogenesis inhibitors such as angiostatin and antiangiogenic antithrombin III (aaATIII) (170). These findings are closely related to the initial hypothesis about the important role of endogenous inhibitors and the existence of a very delicate and finely tuned balance between inducers and inhibitors of tumor angiogenesis.

Many other molecules may be antiangiogenic, and they are currently under active investigation or even in clinical trials. IFN- $\gamma$  was shown to inhibit both endothelial cell proliferation and angiogenesis in vitro, and, furthermore, it had a dramatic effect in the treatment of hemangioendotheliomas. It is assumed to function through modulation of FGFR. Another class of angiogenesis inhibitors that has been derived from fumagillin, an antibiotic purified from fungal cultures, inhibit endothelial cell proliferation in vitro. To avoid toxic effects of the parent compound, AGM-1470/TNP-470, a synthetic analog with enhanced antiangiogenic activity, has been synthesized (171) and is being tested in clinical trials. Protamine, a cationic protein derived from sperm, was shown to be a specific inhibitor of angiogenesis, probably by interfering with growth factors. Platelet factor IV, released from platelets during aggregation, inhibits the growth of solid tumors when used as a recombinant protein. A series of corticosteroids tested in animal models in conjunction with heparin showed effective antiangiogenic activity. They have been termed *angiostatic steroids*. Some inhibitors of the signal transduction from the angiogenic factor receptors, such as genistein or herbimycin, are being investigated as angiogenic inhibitors (172). Even additional natural compounds such as extracts from avascular tissues have been shown to be antiangiogenic, including an inhibitor of neovascularization from cartilage (173).

### Prognostic Value of Angiogenesis

As early as 1972, Brem et al. (174) developed a microscopic angiogenesis grading system with which to quantify the tumor angiogenesis. The goal was to establish an objective method for measuring the tumor vasculature and its relationship to the clinical parameters of the disease. This entailed the search for a useful angiogenic index, not only for its prognostic value, but also to stratify patients for therapy (175). The first results obtained by several groups showed a high level of variation related to the sample selection; inter- and intraobserver variation, owing to the limited experience in vessel counting, and the specificity of the marker used. These limitations delayed the achievement of the goal. Nevertheless, 20 yr later it was shown in breast cancer that microvessel density was an independent prognostic marker for both relapse-free and overall survival. These studies were done using factor VIII to identify the endothelium and established criteria for microvessel assessment (176–178).

Since the reproducibility of the grading system method is poor, several improvements have been proposed, including the use of a microscope eyepiece grid (179).

Other groups are testing more sensitive endothelial markers such as CD31 or CD34. The correlation between tumor angiogenesis and prognosis of lung adenocarcinoma (T1N0M0) has been studied using MAbs to CD31 that showed microvessel count might be a major prognostic factor and a useful tool to predict recurrence in patients with lung adenocarcinoma (180). CD34 has been used successfully on samples from ovarian cancer (181), gastric carcinomas (182), and malignant mesotheliomas (183).

Vermeulen et al. (184) has proposed standardization of angiogenic quantification to reduce interlaboratory variability and to confirm the prognostic value of intratumoral microvessel density in solid tumors. They suggested a detailed standard immunostaining (CD31 marker) method for assessment and predicted the increased role of serum concentrations of angiogenic factors (bFGF, VEGF) as markers of tumor progression. Moreover, new specific markers for activated endothelium (e.g., Abs to endoglin and integrins) are being studied to verify whether the ratio of activated/quiescent endothelial cells could add prognostic information to intratumoral microvessel assessment.

Other investigators have shown positive correlation between tumor neovascularization assessed by IHC staining with anti-CD31 Abs and VEGF mRNA expression in breast tumors (185). Further studies have shown the direct relationship between VEGF expression and tumor angiogenesis in cervical intraepithelial neoplasia and head-and-neck squamous cell carcinoma (186,187). These findings are consistent with those of previous studies that reported the association of VEGF expression with early relapse in bladder carcinomas and its use as an independent prognostic marker in breast carcinomas (188,189).

Despite the discrepancies observed in the literature, mainly due to the criteria used for microvessel counting, the results achieved suggest a significant correlation between high tumor neovascularization and a reduction in patient survival (190). The definitive angiogenic index may be a multiparametric factor instead of a single histologic measure of microvessel density in tumor tissue.

## Therapeutic Approaches

As described, angiogenesis is a complex multistep process in which many potential key points may be susceptible to therapeutic intervention. Our current knowledge of the paracrine talk between tumor and endothelial cells allows us to define specific targets for therapy. Thus, the endothelial mitogens released by tumor cells; the tumor growth factors secreted by endothelial cells; the proteases released by both populations for degrading the local stroma; the pivotal role of some integrins, such as  $\alpha\beta3$  and  $\alpha\beta5$ , in endothelial cell proliferation and migration; and, finally, the natural endogenous angiogenesis inhibitors all contribute to neovascularization and are therefore potential targets for pharmacologic modulation.

The theoretical advantages of antiangiogenic therapy include the expected low toxicity of the specific antiangiogenic agents due to the slow turnover rate of the endothelial cells in normal tissues compared with the turnover of cells involved in tumor angiogenesis, reduction in the risk of developing drug resistance because of the stability of the endothelial cell genome, and the dual effect of the therapy when the target chosen is expressed by both tumor and endothelial cells (e.g., metalloproteinases,  $\alpha\beta3$  expression on melanoma and endothelial cells). This last point is clearly indicated in a recent publication, in which Folkman and colleagues (191,192) have considered two mechanisms, "direct" and "indirect," for angiogenesis inhibitors. The direct angiogenesis inhibitors are the agents that work on the endothelial cell, blocking its

**Table 1**  
**Antiangiogenic Therapy**

Strategy	Agents
Inhibition of endothelial cell proliferation/migration	TNP-470, angiostatin, endostatin, linomide, genistein, interferons, suramin, antibodies against angiogenic growth factors, thrombospondin, angiostatic steroids
Inhibition of proteolytic enzymes	TIMPS, metalloproteinase inhibitors (Batimastat, Marimastat), cartilage-derived inhibitors, plasminogen activator inhibitors, minocycline, tetracycline
Inhibition of cell tube formation and induction of apoptosis	$\alpha v\beta 3$ integrin antagonists: Vitaxin, 17E6, cyclic-RGD peptides, mimetics, interferons, angiostatin, endostatin

locomotion and/or proliferation; and the indirect agents act on tumor cells, inhibiting the production of an angiogenic factor, neutralizing the angiogenic factor itself, or blocking its receptor on the endothelial cell.

Examples of indirect angiogenesis inhibitors are IFN- $\alpha$  and trastuzumab, which inhibit the tumor cell production of bFGF and VEGF, respectively. Another family of antiangiogenic drugs that destroy tumor blood vessels has been described. A representative of the so-called vascular-targeting agents is combretastatin A4, which is currently in phase 2 studies (193).

Kerbel and Folkman (1) have summarized the most advanced antiangiogenesis clinical trials. Some of the compounds tested have shown disappointing results. For example, the clinical trials with SU 5416 were stopped after it failed to meet its end points in colorectal cancer. Avastin, the anti-VEGF MAb, did not meet its primary end point of progression-free survival in a phase 3 breast cancer trial. Although preliminary, the first endostatin clinical trial outcome has been defined as lukewarm by specialists (194). These preliminary, unsuccessful examples point out that it is very important to consider two aspects for the future development of antiangiogenic therapy: first, the establishment of specific treatment protocols for these types of agents; and, second, the definition of valid surrogate markers that allow measurement of the antiangiogenic activity of the drugs during treatment. Regarding the first issue, it is necessary to emphasize that frequent and low dosing of some current chemotherapeutic drugs, such as vinblastine, have shown antiangiogenic activity and improved the control of tumor growth (195). This antiangiogenic chemotherapy schedule has been called metronomic therapy (196,197). Therefore, the design of new clinical protocols for antiangiogenic therapy is mandatory. With respect to the second issue, the measurement of surrogate markers such as VEGF, VCAM-1, and circulating endothelial cell progenitors (CD133+, VEGFR-3+) seems to be the optimal marker for following antiangiogenic therapy.

Current preclinical and clinical research can be summarized in the following approaches: inhibition of endothelial cell proliferation/migration, inhibition of the proteolytic enzymes involved in the ECM degradation, and inhibition of cell tube formation and induction of apoptosis. Table 1 summarizes these approaches and the agents involved, while Table 2 provides some of the most representative antiangiogenic clinical trials. For a detailed review of the antiangiogenic compounds, see refs. 1, 152, and 198–200.

**Table 2**  
**Angiogenesis Inhibitors in Clinical Trials of Cancer**

Drug	Sponsor	Mechanism	Phase
Neovastat	Aeterna	Prevents VEGF binding to its receptors; inhibits MMP-2, -9, -12	2/3
Thalidomide	Cewlgen	Unknown; inhibits TNF- $\alpha$	2/3
BMS-275291	BMV/Celltech	Second generation of MMP inhibitors	1/2
Endostatin	Entremed	Unknown; disrupts actin cytoskeleton	1/2
Avastin	Genentech	MAb against VEGF	2/3
Vitaxin	Medimmunoe	MAb against $\alpha\beta 3$	1/2
L-651582	Merck & Co	Calcium channel inhibitor	2/3
Cilengitide	Merck KgaA	$\alpha\beta B$ Inhibitor	1/2
PTK-787	Novartis	VEGRF tyrosine kinase inhibitor	2
Combretastatin A4	Oxigene	Unknown; inhibitor of tubulin polymerization	1/2

### ***Inhibition of Endothelial Cell Proliferation/Migration***

TNP-470 is an analog of fumagillin, a naturally occurring antibiotic produced by the fungus *Aspergillus fumigatus*. The antiproliferative mechanism of TNP-470 in the endothelial cell is unknown, but it appears to affect the late G1-phase, inducing an arrest by a potential inactivation of cyclin-dependent kinases that phosphorylate the retinoblastoma protein, pRb. TNP-470 has been tested in phase 1 trials in patients with refractory solid tumors and in patients with acquired immunodeficiency syndrome who have Kaposi's sarcoma. Phase 2 trials in a variety of tumors and phase 3 trials in pancreatic cancer are under way (201). It has been shown that TNP-470 upregulates the expression of prostate-specific antigen (PSA) in patients with androgen-independent prostate cancer, making clinical utilization of this marker a problematic (202). To avoid toxic effects of the parent compound, AGM-1470/TNP-470, a synthetic analog with enhanced antiangiogenic activity, has been synthesized (171) and is being tested in clinical trials. Carboxyamidotriazole inhibits the influx of calcium into cells and inhibits tumor growth and angiogenesis. This compound is being tested in phase 1 trials (203).

Since VEGF emerged as a key growth factor in angiogenesis, several therapeutic approaches have attempted to inhibit angiogenesis by blocking either VEGF, VEGFR, or the signaling induced by VEGF-VEGFR interaction. Thus, high-affinity MABs against VEGF were used in models of tumor growth in vivo. Such Abs were found to exert a potent inhibitory effect on the growth of at least three human tumor cell lines in vivo (204) without affecting the growth of the tumor cells in vitro. At present, the humanized MAb Bevacizumab (anti-VEGF) is being tested in clinical phases 2 and 3. Further studies showed the efficacy of this strategy in distinct in vivo models (205,206). Similar therapeutic approaches focused on the receptor instead of the growth factor. In an elegant study, dominant negative receptors (flk-1) were delivered to the endothelium by retrovirus, to inhibit the growth of glioblastomas multiforme and other tumors in vivo (207,208). Other potent inhibitors of tumor angiogenesis are small synthetic molecules designed to block signaling after VEGFR engagement. A clear example of this kind of angiogenesis inhibitors is the compound SU6668, which blocks tyrosine phosphorylation under the signaling of VEGFR, FGFR, and PDGFR (209). This compound is in clinical trials, although it is important to note that some clinical pitfalls were reported using precursors with perhaps less activity (210). While the VEGF therapeutic approach seems to be effective, the FGF approach is not as clear. Abs to FGF or its receptors gave conflicting results (211), indicating that further studies are needed. Finally, Suramin, a polyanion that disrupts binding of FGFs to their receptors, has been used in murine models with promising results and is being studied in phase 1 trials (212).

This approach could also include the known endogenous inhibitors of angiogenesis such as angiostatin and endostatin, which are being tested in clinical trials in phase 1 and phase 1/2, respectively (213,214). These compounds not only inhibit angiogenesis, but also maintain sustained dormancy of tumors and metastasis by means of an increase in the apoptotic rate in tumor cells (215,216). Experimental antiangiogenic therapy using endostatin revealed no acquired drug resistance (217), in contrast to standard chemotherapy. This work has induced new expectancies, and the concern for an antiangiogenic therapy in cancer has increased since its publication. Folkman's group has proposed a novel antiangiogenic gene therapy with either angiostatin, endostatin, or a fusion protein of both molecules (218,219). Again, the antiangiogenic



therapy showed antitumor effects through the maintenance of the dormant status in micrometastasis (218). Although studies using angiostatin and endostatin resulted in more promising preclinical data in the field of antiangiogenic therapy, concerns about their activity remain and some investigators have published negative results in terms of no activity (194).

### ***Inhibition of Proteolytic Enzymes***

Proteinase inhibitors can block proteolytic activity of both activated endothelial cells and migrating tumor cells. Both natural inhibitors (TIMPs) and synthetic inhibitors of MMPs have been described as potential antiangiogenic and antimetastatic drugs. The most exciting progress has been made in the field of synthetic inhibitors of MMPs.

The first generation of synthetic MMP inhibitors is represented by the compounds known as Galardin and Batismastat (142). Both agents have poor oral bioavailability, but phase 1 clinical trials of Batismastat used the intraperitoneal (ip) route in patients with malignant ascites, or the intrapleural route in patients with malignant pleural effusions. A new generation of orally active MMP inhibitors includes the compound Marimastat, which has been tested in phase 2 clinical trials in 232 patients. The oral bioavailability is very good and the main side effects observed were myalgias and arthralgias of unknown origin. No clinical efficacy was shown using either of the initially promising new inhibitors. The failure of MMP inhibitors to alter disease progression in metastatic cancer might have been anticipated since MMPs appear to be important in early aspects of cancer progression (local invasion and micrometastasis) and may no longer be required once metastases have been established (16). New investigations have led to a better understanding of the role of MMPs in tumor biology and angiogenesis. They have a dual function in tumor angiogenesis: some MMPs are required to break down basement membrane barriers in the early stage of angiogenesis, while other MMPs are involved in the generation or release of proangiogenic and antiangiogenic molecules.

### ***Inhibition of Cell Tube Formation and Induction of Apoptosis***

A third approach disrupts vessel formation and induces endothelial cell apoptosis. Many antiangiogenic compounds are in this category and have been reviewed in previous sections. This section therefore focuses on inhibitors of  $\alpha v\beta 3$  integrin.

Antagonists of  $\alpha v\beta 3$  prevent blood vessel formation in several in vitro and in vivo models of angiogenesis (24,91,100). This angiogenesis blockade results in tumor growth modulation and, eventually, in a regression of preexisting  $\alpha v\beta 3$ -negative human tumors (96,99). Moreover, because of the null expression of  $\alpha v\beta 3$  in quiescent vasculature, the antagonists do not affect normal blood vessels and are unlikely to cause toxic effects or side effects. Most of these studies were done using an MAb directed to the  $\alpha v\beta 3$  heterodimer LM609 (100), an MAb directed to the  $\alpha v$  subunit 17E6 (106,107,109) and recognizing both  $\alpha v\beta 3$  and  $\alpha v\beta 5$  (97), or cyclic penta- or heptapeptides containing the adhesive Arg-Gly-Asp sequence (98). In particular, the use of low-molecular-weight molecules such as cyclic Arg-Gly-Asp peptides is of great interest because of the relative ease with which they reach the tumor vessels and because they are synthetic compounds, with the consequent advantages of low production cost. Some researchers used a mixed approach to obtain novel antagonists of the VNR. Using phage display technology, several groups have created libraries of single-chain Abs with loops containing heptapeptides (25). In this way, highly active new sequences were identified. Furthermore, those constructions when tested in vivo

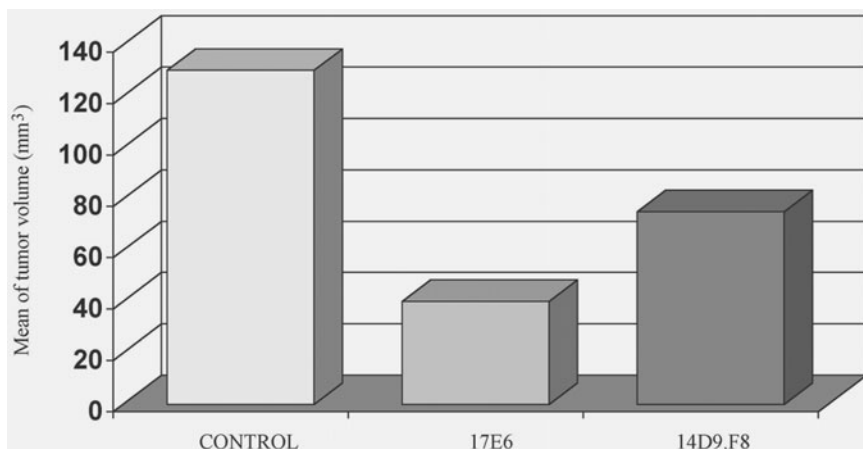


Fig. 3. Normal human skin was grafted onto SCID mice. After wound healing, M21-L human melanoma cells (negative for  $\alpha v\beta$ ) were intradermally injected into the human dermis. Therapeutic drugs were MAbs 17E6 and 14D9.F8 (anti- $\alpha v$ ). For further details see text and refs. 106 and 107.

were targeted to the tumor blood vessels (25). LM609 has been humanized (Vitaxin) and clinical trials are ongoing. Our group has developed the aforementioned anti- $\alpha v$  17E6 MAb as well as cyclic Arg-Gly-Asp peptides, which have shown a potent inhibition of both  $\alpha v\beta 3$  and  $\alpha v\beta 5$  targets (99,106,107) (Fig. 3). The preclinical research done on both compounds confirmed their effectiveness as antiangiogenic agents, and, thus, clinical trials of the cyclic Arg-Gly-Asp peptide (EMD 121974) (Cilengitide) have commenced, beginning currently in phase 2 trials.

## Conclusion

The progression of tumors to malignancy and the establishment of metastasis depend on the induction of neovascularization. Cells in a developing tumor will progress only if they acquire the angiogenic phenotype necessary to attract the new vessels on which their malignancy depends. We have reviewed the distinct pathways involved in such a process, referred to as the angiogenic switch. One key step is the delicate balance between natural inducers and inhibitors of angiogenesis. The tipping of this balance toward one side or the other side would favor the inhibition of angiogenesis or promote neovascularization. Current antiangiogenesis research is pursuing novel, potent inhibitors, which may lead to new therapeutic drugs for cancer treatment.

The preliminary clinical research with the first generation of antiangiogenic agents has yielded some disappointing results. This fact, considered together with the growing increase in the knowledge of the basic mechanisms underlying tumor angiogenesis, has at least three interpretations. First, basic research must be transferred to clinical research. Kerbel and Folkman (1) reviewed this issue and highlighted this crucial need. Second, the design of clinical trials should consider the clinical evolution of the selected target. This means that therapies addressed to very specific molecular targets need a very careful selection of both the tumor indication and the patient population.

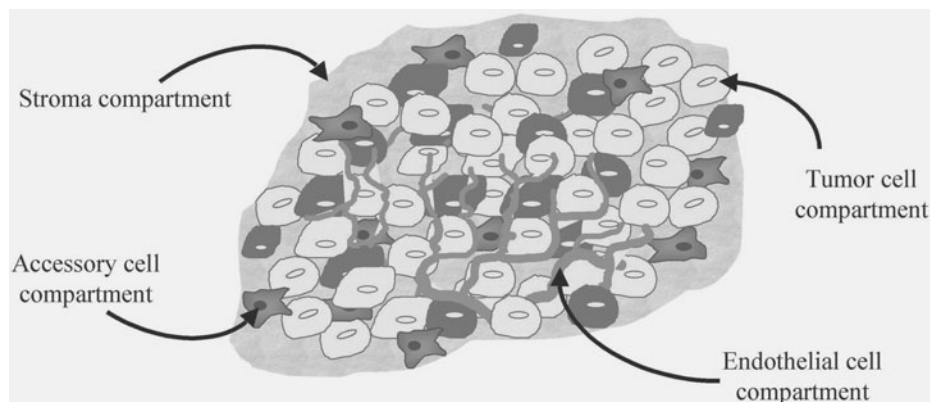


Fig. 4. Model of combined therapy. Within a tumor, four compartments can be distinguished: the tumor cells, the endothelial cells, the accessory cells, and the stroma. An antitumor therapy (affecting the proliferative rate of tumor cells) combined with an antiangiogenic therapy (affecting both the endothelial cell population and the apoptosis rate of the tumor cell population; see text) would lead to a more effective anticancer global therapy. This combi-therapeutic approach might also be accomplished and eventually increased by targeting all four compartments within a given tumor.

This way, the new drug would have a more realistic pharmacologic potential. Third, perhaps antiangiogenesis therapeutic intervention, given as single agents, is not the best way to control tumor growth and metastatic dissemination. Thus, this idea leads to an adjuvant treatment for controlling cancer diseases. Antiangiogenic therapy would enhance the action of both the classic cytotoxic chemoradiotherapy and the new immunotherapy in cancer patients due to the action on the different compartments within a tumor, including endothelial, tumor and accessory cell populations, as well as the stroma compartment (220) (Fig. 4). Therefore, this approach may mimic the endogenous inhibitors of angiogenesis, such as angiostatin, maintaining tumor dormancy. Consequently, this therapy could facilitate a strategy that tends toward a chronicity of the disease in a way similar to the treatment of diabetes and AIDS.

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# 13

## Invasion and Metastasis

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**Maria Rosa Bani and Raffaella Giavazzi**

### **Cancer Malignancy and Metastasis**

Metastasis is the most life-threatening aspect of malignant neoplasm. When a tumor remains localized, its surgical removal generally results in the survival of the patient and, thus, the lesion is called benign. By contrast, when a tumor invades adjacent tissues and spreads to other anatomic sites, the possibility of cure becomes poor and the lesion is called malignant. Cancer can disseminate by hematogenous spread (e.g., sarcomas), by lymphatic spread (e.g., carcinomas), or by seeding within natural body cavities (e.g., ovarian carcinoma within the peritoneal cavity). The lymphatic and venous systems are highly interconnected, allowing disseminating cancer to pass from one system to the other. Clinical and pathologic observations point to the local/regional lymph node spread as an early occurring event. Use of the sentinel lymph node is a promising staging method in the diagnoses of breast carcinoma and melanoma (1,2). Anatomy by itself does not fully explain the distribution of metastasis in different organs (3). The dissemination of cancer is a complex process and the outcome depends on numerous interactions between the cancer cell and the host environment (Fig. 1). During malignant progression, tumor cells must elude the immunosurveillance mechanisms and lose their responsiveness to the normal growth controls. To successfully develop metastatic foci, cancer cells detach from the primary tumor mass; penetrate the basement membrane and invade the surrounding host stroma; gain access to blood or lymphatic vessels by crossing subendothelial basement membrane and survive in the circulation; arrest and extravasate through the vessel walls; infiltrate the surrounding host tissue compartments; and finally grow in the newly colonized organ (4–8). The process is dynamic and the diverse steps can take place at different times. The clinical patterns are different as well. When the primary tumor is first diagnosed, metastases may be detectable or may be present but not detected (occult). In the latter scenario, after removal of the primary lesion, metastases may appear shortly afterward. Alternatively, they may take a very long time to become detectable.

It is clear that the sole unrestrained ability to grow does not result in metastasization, but that a variety of cellular processes are needed. Particularly relevant is the concept that the growth of solid tumors is largely dependent on the formation of new blood vessels (angiogenesis) (9,10), especially during the early phases of tumor growth

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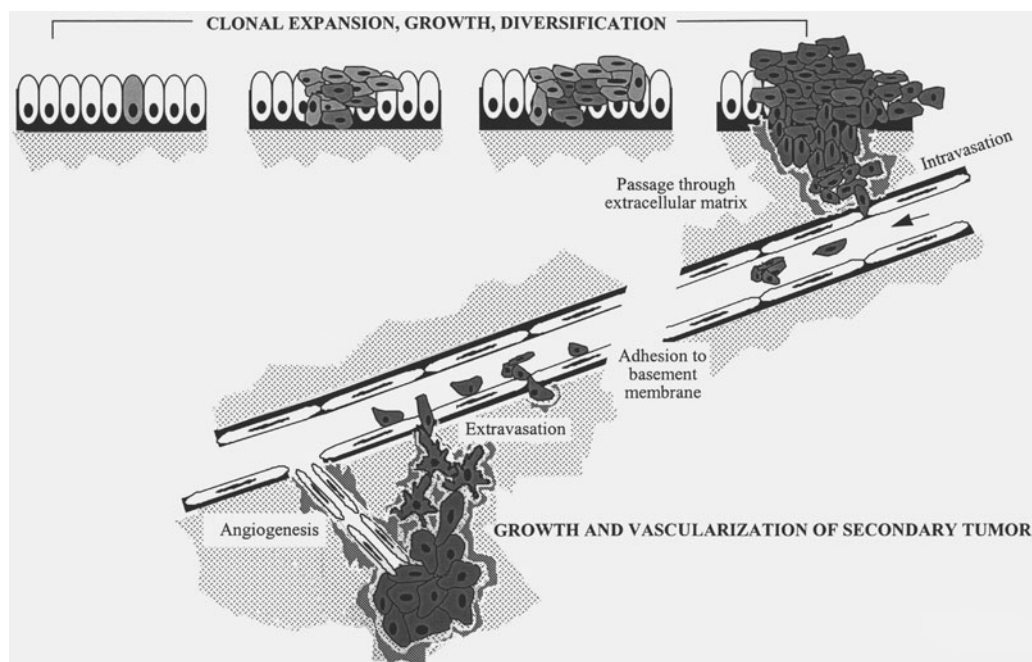


Fig. 1. Schematic of cancer malignancy. Cancer is a multistep process occurring as a result of genetic and epigenetic alterations associated with phenotypes leading to tumor formation and spread of metastasis. Metastasis involves an intricate interplay among cell adhesion, proteolysis, migration, and angiogenesis.

(11,12). Vascularization has an important role in controlling the expansion of the metastatic foci in the secondary sites (13–19). Furthermore, high angiogenesis activity and vascular remodeling appear to be important requirements for metastasis that do not require invasion through the vascular wall (20). The formation of lymphatic vessels (lymphoangiogenesis) has received particular attention in the last few years, on the discovery of lymphoangiogenic factors and the observation of their expression by human cancers. Several experimental models suggest that the capacity of a tumor to induce lymphoangiogenesis may be critical for determining the metastatic potential of cancer (21–24).

### Phenotype of the Metastatic Process

Given the complexity of the cancer development process, it appears that multiple changes contribute to its malignancy. Genomic instability generates heterogeneity resulting in cells with new phenotypes; unknown microevolutionary selection pressures then allow the progressive selection of those cells endowed with enhanced malignant potential (25–27). The continuous emerging of cell populations with different abilities to invade and form metastasis represents a major obstacle to therapy (28). Although a series of defined biologic abilities and phenotypic features are necessary (Fig. 1), new and different genetic and epigenetic determinants may occasionally be described in malignant cancers (29,30). It is expected that the strongest candidate molecules for predicting disease outcome and metastasis are those involved in cellular adhesion and extracellular

matrix (ECM) degradation, or able to activate blood and lymphatic endothelial cells, stromal cells, and tumor cells themselves.

### **Adhesion**

The initial step in the metastatic cascade is the detachment of cells from the primary tumor mass. It appears that, contrary to benign lesions, in malignant tumors, the loss of cohesiveness renders the cells easily detachable from the main tumor mass. For example, E-cadherin normally mediates adhesion of epithelial cells through adherens junctions, and it is the main factor responsible for cell–cell attachment in epithelial tumors. Immunohistochemistry (IHC) studies showed that loss or reduction of E-cadherin expression correlated with the clinical stage in a broad range of malignancies, including cancers of the head and neck, lung, breast, prostate, esophagus, stomach, bladder, pancreas, and colorectum (31,32). A multitude of experimental results show a strong association/correlation between invasiveness and deregulated function of E-cadherin (33,34). Accordingly, in a transgenic mouse model of pancreatic  $\beta$ -cell carcinogenesis, loss of E-cadherin coincided with the transition from differentiated adenoma to invasive and metastatic carcinoma (35). The extracellular portion of E-cadherin is responsible for the homophilic interaction of neighboring cells, while the cytoplasmic portion interconnects with the intracellular environment through catenins. By interacting with transcription factors and mediating signaling to the nucleus, catenins may have roles other than simply modulating cell adhesion. For example, the APC protein (the product of the adenomatous polyposis coli gene) that binds and modulates  $\beta$ -catenin degradation contributes to regulation of the cellular level of  $\beta$ -catenin. In cells where APC is mutated, the amount of free  $\beta$ -catenin increases, its interaction with transcription factors is favored, and gene expression is modulated (36). In addition, mutation in  $\beta$ -catenin itself can activate such a signaling pathway (37,38). Furthermore, it has been shown that abrogation of E-cadherin function leads to upregulation of urokinase-type plasminogen activator (uPA), a matrix-degrading enzyme (39), thus suggesting a possible signaling pathway connecting adhesion and proteolytic degradation. These observations indicate that the cadherin/catenin complex on tumor progression and metastases have a dual influence: modulation of homophilic cell adhesion and direct signaling to the nucleus (36,37,40,41).

As for cadherins in epithelial-type cancers, the metastatic ability of some lymphomas correlates negatively with the expression of the neural adhesion molecule L1 (NgCAM) (42). As another example, the *DCC* (*deleted in colorectal carcinoma*) expression is absent or markedly reduced in most colorectal cancers and in several tumor types (43). *DCC* encodes a transmembrane protein product with sequence similarity to the neural cell adhesion family of molecules; thus, the loss of function of this gene may affect adhesive interactions. Again, it is tempting to speculate that the loss of an adhesion pathway could be an important step for the spread of certain tumor types (44).

Other alterations may take place in the adhesive events that orchestrate the release of neoplastic cells and their access to the underlying substrates. Attachment to specific glycoproteins of the ECM is mediated through cell receptors. For example, integrins are a large class of  $\alpha\beta$  heterodimeric receptors with broad specificity and of great importance in cell–substrate interactions. Ligand specificity is determined by the  $\alpha\beta$ -subunit composition, although the same ligand can bind to more than one integrin and the same receptor can bind more than one ligand. Differences between normal and malignant tissues have been documented for many tissue types (45). Generally,



integrins appear to be downregulated in the more advanced stages of naturally occurring cancers. Studies on melanoma and carcinomas of the breast, pancreas, colon, and lung have shown the involvement of  $\alpha 1$ ,  $\alpha 6$ ,  $\beta 1$ , and  $\beta 4$  integrin, suggesting that loss of attachment to the basement membrane, which is mainly composed of laminin and collagen IV, is an important event in favoring malignant progression. Several studies examining the biologic effects as a consequence of integrin expression confirm their role in modulating metastasis and support their downregulation as one of the de-adhesion mechanisms favoring metastasization (46,47).

We have so far described how downregulation of certain molecules contributes to metastasis by favoring tumor cell detachment from the primary lesion. Yet, the first step in the invasion process involves the adhesion of tumor cells to the matrix. Accordingly, not all cancers manifest a generalized downregulation of integrin expression. For example, the expression of  $\alpha 4\beta 1$  integrin was described as negatively associated with the length of disease-free interval and the overall survival time in cutaneous malignant melanoma (48). The integrin  $\alpha v\beta 3$  was found strongly expressed on the invasive front of malignant melanoma while it was poorly expressed in preneoplastic lesion. The forced expression of  $\alpha v\beta 3$  in a melanoma cell line increased its metastatic potential (49). Many investigators have shown that adhesive interactions mediated by integrins are among the necessary requirements for metastasis formation. When integrin/ligand interaction was hampered by different means in a variety of tumor types, the formation of experimental metastasis was inhibited (50–53).

Hence, the relationship between integrin expression and cancer appears to be complex. It is likely that cell–matrix adhesion events may be required at certain times and in certain locations whereas they may impose constraints in others. During migration, cells are constantly breaking and making integrin contacts. Cells remain immobile if the adhesion is too strong, but they cannot generate enough traction to move if the adhesion is too weak. Thus, in response to intracellular signals, integrins change in the way of interacting with the extracellular environment. Such inside-out signaling is necessary to modulate the level of adhesiveness. By regulating integrins' relocation and their ligand affinity and avidity, the binding to the extracellular components is altered without affecting the level of gene expression.

Integrins are involved in regulating the activities or proteolytic enzymes that degrade the basement membrane (54). During invasion, the integrin  $\alpha v\beta 3$  acting in concert with a multiprotein complex contributes to its localization at the invasive front and to the activation of protease required for the degradation of ECM (55). Integrins not only mediate adhesion, but such transmembrane proteins create a structural link between components of ECM and the cytoskeleton (56), and are able to transduce signals to influence diverse cellular functions such as apoptosis, survival, proliferation, cell shape, and ultimately invasion and migration (54,57–59). The signaling pathways typically involve the phosphorylation of focal adhesion kinase (FAK)—a cytoplasmic protein that colocalizes with integrins—the recruitment of adapter proteins; the action of small guanosine 5'-triphosphatases (GTPases), and the activation of the downstream effector molecules. For example, activation of RAS-ERK (ras-extracellular signal regulated kinase) pathway promotes cell proliferation. Recruitment of SCR contributes to cell motility, while the differential recruitment and activation of the SHC adapter proteins affects how cells migrate. Activation of the small G protein CDC42 is involved in the formation of filopodia, while RAC activation promotes actin reorganization and contributes to membrane ruffling. In

some instances, phosphatidylinositol 3' kinase activation is required for CDC42- and RAC-mediated invasion and migration of cancer cells. In addition, RAC and CDC42 potentiate ERK signaling and increase cancer cell responsiveness to growth factors. Binding of integrins to ECM components initiates prosurvival mechanisms to prevent apoptosis. Notably, multiple signaling pathways involved in FAK mediate prosurvival are those also implicated in migration (54–59).

Several other adhesive interactions involved in various cell–cell heterophilic interactions have been implicated in metastatic spread, and the upregulation of certain molecules has been shown to correlate with a higher potentiality of tumor cells to metastasize. For example, the intercellular adhesion molecule-1 (ICAM-1) has been shown to be a marker of progression in malignant cutaneous melanoma (60,61). It is possible that as ICAM-1 binds to  $\beta 2$  integrins expressed on circulating leukocytes, an indirect interaction with leukocytes may mediate the binding between tumor cell and endothelium allowing enhanced extravasation. Vascular cell adhesion molecule-1 (VCAM-1), the ligand for  $\alpha 4\beta 1$  integrin, is constitutively expressed on bone marrow stromal cells. For this reason, it has been hypothesized to be partly responsible for the retention of leukemia cells in the marrow and bone marrow lymphoma metastases (62). Increased amounts of circulating forms of ICAM-1 and VCAM-1 have been found in patients with cancer (63–65). Whether the increased level actively contributes to the progression of malignancies remains to be determined.

### **Interaction With the Vasculature**

To form distant metastatic foci, circulating cancer cells arrest in the vascular bed of the target organs, and then eventually extravasate and grow in the new environment. Studies using intravital videomicroscopy indicate that most circulating cells arrest in small capillaries by size restriction. Cancer cells undergo adhesive arrest in the larger precapillary vessel (venules) (66). It has been proposed that cancer cells may start to proliferate without necessarily extravasating. Indeed, the existence of intravascular tumor colonies has been reported (67–69). Recently, in an experimental model of pulmonary metastases, it has been shown that cells first arrested in the subpleural microvasculature and firmly attached to the endothelium; second, they proliferated originating micrometastases entirely contained within the vasculature that later outgrew the vessels (70).

Vascular changes can influence the interaction of tumor cells with endothelium. Chemotactic factors released from the vascular wall induce tumor cell motility (71,72), while the formation of platelet-fibrin thrombi influences the arrest of tumor cells (73,74). Furthermore, the adhesion of tumor cells to vascular endothelium can be modified in response to inflammatory cytokines and growth factors that affect their adhesive properties (75,76). Increased numbers of metastatic foci have been shown in mice treated with interleukin-1 (IL-1) or tumor necrosis factor (TNF) (68,77). These types of adhesive interactions mediated by selectins and integrins have been well described. The enhanced binding of colon carcinomas to cytokine-activated endothelial cells appeared mainly mediated by E-selectin. Although a variety of tumor cells display E-selectin-dependent *in vitro* adhesion to endothelial cells, the role of this interaction in metastasis is often confusing (78). Under dynamic flow conditions, rolling on the surface of the endothelial cells preceded the adhesion of several types of carcinoma cells, and E-selectin mediated this interaction. At variance, melanomas adhered firmly, without rolling, and their firm adhesion was partly blocked by anti-VCAM-1 antibody (79).

The presence of the carbohydrates sialyl-Lewis<sup>X</sup> (sLe<sup>X</sup>) and sialyl-Lewis<sup>a</sup> (sLe<sup>a</sup>), the selectins' ligands, found on several carcinomas, contributed to tumor cell arrest in secondary organs and metastasis formation (80,81). Melanoma cells expressing VLA-4 ( $\alpha 4\beta 1$  integrin), the ligand for VCAM-1, were able to produce more lung colonies in cytokine-treated mice. Treatment of these tumor cells with monoclonal antibodies (MAbs) against  $\alpha 4\beta 1$  inhibited this increase (51,82).

### **Invasion**

As the tumor mass grows within tissues, at either the primary or secondary site, invasion of the surrounding tissue must take place. To form distant metastases, the blood vessel walls must be breached twice: during intravasation first and then during extravasation of the metastatic cancer cell. Therefore, proteolytic degradation of ECM and subendothelial basement membrane components are necessary requirements.

At variance with benign lesions, this implies that malignant cells might be able to traverse adjacent tissues more readily by acquiring such ability. Alternatively, they might take advantage of proteolytic activities derived from host tissue components, including stromal and infiltrating immune cells. For example, spots of gelatinase activity have been observed at the tumor stroma interface. Moreover, IHC studies in tissue sections have shown positive staining for matrix metalloproteinases (MMPs) limited to tumor cells in some cases and associated with stromal tissues in others (83). Indeed, we have shown that gelatinases (MMP-2 and MMP-9) observed *in vivo* in a model of endothelioma originated from the recruited stromal cells rather than from the cancer cells themselves (84). Such findings suggest once more that the tumor environment is indeed an integral part of the malignant disease. MMPs are active against all the components of the ECM and the basement membrane, and they are capable of cleaving fibrillar collagen at physiologic pH. Secreted MMPs are inactive zymogen (proMMP) and require extracellular activation. The storage of MMPs in secretory granules (85) is another way to amplify the degradation processes. The regulation of enzymatic activity is complex, and it is balanced between the local concentration of the endogenous activators and inhibitors. Any of them could be responsible for malignant progression and dissemination. Tissue inhibitors of metalloproteinases (TIMPs) inhibit metalloproteinase activity by forming a complex with MMP. Endogenous molecules such as growth factors and inflammatory cytokines can modulate the activity of both MMP and TIMP, often in opposite ways (86). Extensive literature reports link the association of MMP and TIMP family members with malignancy. Several *in vivo* models have shown the overexpression of TIMPs associated with a reduction in experimental metastasis (87–90). Inhibition of MMP activity may not necessarily prevent the extravasation of cancer cells, as suggested by the observation that cancer cells overexpressing TIMP-1 will leave the bloodstream as efficiently as parental cancer cells (91). It may, instead, contribute to the prevention of growth within the secondary organ, as the metastatic ability of the TIMP-1 cells was greatly reduced (91). Such a phenomenon might be mediated through the inhibition of angiogenesis (a process in which MMPs play critical roles) and by this means contribute to keeping the metastasis in a dormant state.

Despite these earlier observations in favor of TIMPs as blockades of invasion and metastasis (92), recent observations indicate that TIMPs may act in favor of tumor growth (93,94). One of the paradoxes is the finding that TIMP-2 (recognized as a potent inhibitor of MMP-2) functions as an adapter required for proMMP-2 activation

at the cell surface (95,96). Notably, some angiogenesis inhibitors may result from proteolytic cleavage of molecules implicated in other functions. For example, angiostatin is a 38-kDa product generated by cleavage of plasminogen (97) and endostatin is an 18-kDa product derived from collagen XVIII (98). They have both been described to inhibit metastasis formation. Their generation may require MMP activities (99–102), and TIMP may have a positive role in angiogenesis by preventing their production. Together, these findings may have profound implications in designing cancer therapies based on MMP inhibitors (103).

With the exception of TIMP-3 (104), TIMPs have growth promotion and anti-apoptotic effects in many experimental settings (105,106). Increased TIMP expression has been seen in a variety of cancers and is associated with poor prognosis. For example, preoperative high concentrations of plasma TIMP-1 were associated with shorter survival in patients affected by colorectal, lung, gastric, and ovarian carcinoma (107–110). In accordance, high amounts of TIMP-1 and TIMP-2 mRNA in the primary carcinomas were strongly associated with the development of metastases in patients with breast cancer (111).

MMPs are not the only proteolytic contributors to tumor progression. A broad range of proteolytic enzymes have a role in the invasiveness of tumor cells (112). The lysosomal enzymes cathepsins are expressed in various types of cancer. These enzymes can actively degrade ECM, but they can also activate proMMPs (113). Thus, the interactions between members of different classes of proteolytic enzymes might provide additional levels of complexity and regulation of the invasion mechanisms (114–118). uPA and its receptor system (uPA/uPAR), generate PA-active enzyme able to catalyze the conversion of plasminogen into plasmin. Plasmin can then either directly degrade the target proteins or activate the proMMP. In addition, membrane-bound plasmin is responsible for PA activation occurring on pro-uPA binding to uPAR. The enzymatic activity of PA is inhibited by plasminogen activator inhibitor (PAI) family members. uPAR contributes to the adhesion of cancer cells to ECM, by enhancing the binding to vitronectin. In such a context, PAI-1, independently from its function of proteinase inhibitor, interferes with cell migration by competing with the binding of vitronectin to integrins (119,120). IHC studies have shown that components of the uPA/uPAR system are localized both at the invasive front of the tumor and in the stromal tissue (121). Once again, the complexity of the interactions existing between tumor and host during the invasive process is illustrated. Matrix degradation is essential for the invasion of tumor cells (metastasis formation) and of the endothelial cells (vessel formation) (9). During the angiogenesis process, MMPs and uPA/uPAR are involved in the remodeling of ECM that must accompany the formation of the new vessels, which, in turn, are necessary for the growth of tumors and metastatic spread.

### **Motility**

Tumor cells are capable of active movements through tissues, and individual or small aggregates of tumor cells have been found physically separated from the main tumor mass. A variety of agents, including components of ECM and host-derived and tumor cell-secreted factors, can generate signals that modulate the motility of cells (122–125). Such a variety of stimuli may provide multiple opportunities to cells to move across the different environments during the metastatic process. How motility factors regulate cell movement is not fully known. The transduction of signals after ligand–receptor coupling is likely to be important in the regulation of cell locomotion.

Hepatocyte growth factor/scatter factor (HGF/SF) directly stimulates cell motility (126), but it also alters the tyrosine phosphorylation of the cadherin-associated  $\beta$ -catenin (127,128), which may, in turn, impair cadherin's adhesive functions (129). Finally, HGF/SF mediates proteolysis by upregulating the expression of uPA and uPAR (130), and of MMP (131,132). Hence, through a focal degradation of ECM, it may favor invasion. Together, these findings describe the potential ability of HGF/SF to promote multiple cellular programs for metastasizing cells (133). Indeed, HGF/SF not only is present at high concentrations in the stroma of malignant tumors, but it also has been found in the axillary lymph node of invasive breast carcinoma and in the pleural effusion of cancers metastatic to the lung (134,135).

Another example is autotaxin (ATX), which was isolated as a tumor motility-stimulating secreted protein (125) and whose ability to stimulate cell motility has been described for human melanoma, neuroblastoma, and breast and prostate carcinoma cell lines (136–139). ATX is expressed in melanoma, teratocarcinoma, and neuroblastoma cell lines (138–140), and greater expression was seen in hepatocellular carcinoma than in normal liver tissue (141). ATX expression augmented in vitro invasiveness as well as in vivo metastatic potential of tumorigenic ras-transfected NIH3T3 cells but did not affect aggressiveness and malignancy of the non-tumorigenic NIH3T3 cells despite enhancing their in vitro invasiveness (142). Notably, at concentrations similar to those that induce motility and invasion, ATX affects endothelial cell tubule formation and smooth muscle cell migration (143). As both a tumor motogen and an angiogenic factor, ATX appears to have the capacity to stimulate multiple features of the metastatic cascade.

The selective migration of cancer cells to secondary sites appears to determine the organ preference of metastasis (8). It has been reported that chemokine receptors may contribute to the organ preference of migrating cells. For example, breast cancer cells were found to express CXCR4 and CCR7 at high levels and the specific ligands for these receptors were found highly expressed in organs to which breast carcinomas generally metastasize. In a mouse model of breast cancer, the blockade of CXCR4 inhibited metastasis to regional lymph nodes and lung (144).

### ***Dormancy of Metastases***

Once metastatic cancer cells have successfully reached a target organ, they must grow to a certain size to become clinically detectable metastases. Hypotheses about the mechanisms of dormancy of metastases include arrest of tumor cell proliferation, host immunosurveillance, and hormone dependency. One recent hypothesis is that suppression of angiogenesis is responsible for dormancy (16,145–149). For example, in a model of Lewis lung carcinoma (3LL), it has been shown that although microscopic metastases were present in the lung of the mice, they remained avascular and did not expand as long as the primary tumor was present. After surgical removal, metastases became vascularized and expanded to a macroscopically visible, hence detectable size (97).

Angiogenesis may result from an imbalanced availability of proangiogenic factors and angiogenesis inhibitors (150). To this end, the angiogenesis inhibitor angiostatin was isolated from the urine of mice carrying the primary tumor (3LL), and it was then shown to be responsible for metastases dormancy (151). This angiogenic theory for dormant metastasis has opened new perspectives in clinical oncology (152). It has been observed that when no metastatic disease is detectable at the time of the primary tumor diagnosis, different follow-ups can occur:

1. Metastases become detectable in 3% of patients within 2–12 mo after removal of the primary lesion. This suggests that metastases were present at time of surgery, but their expansion was inhibited until the primary lesion was present. According to the model described above, it is possible to speculate that the presence of a circulating angiogenesis inhibitor generated by the primary tumor tilts the balance toward suppression of metastasis growth (152).
2. Metastases take a very long time (years) to develop, as in the case of mammary and prostate carcinomas. Again, this suggests that metastases were present at time of surgery, but in this case one can speculate that the metastatic cells are not able to induce and sustain angiogenesis in the newly colonized organ. They will eventually acquire such ability if given enough time (152).
3. Metastases will never develop. Either metastases were not present at the time of surgery or, if present, they may have regressed or remained dormant during the patient's lifetime. In the latter cases, the growth of metastatic cells may be inhibited by factors present in the new environment, their expansion might need factors not available in the new environment, or they will never acquire the capacity to evoke angiogenesis (152).

## Molecular Genetics of Metastasis

Two classes of genes are the principal targets of genetic alterations: oncogenes, whose action is considered dominant because, on activation, they act despite the presence of their normal counterpart allele; and tumor suppressor genes (TSGs), whose effect can take place only if both alleles are inactivated. Malignant progression involves genetic alterations whose accumulation leads to permanent phenotypic changes (26). Although the loss of TSGs and/or the activation of oncogenes has been consistently associated with malignancy (29,153), what remains to be defined is which of the biologic properties associated with advanced stage disease are causative, strictly associated, and/or simply coregulated with malignancy.

DNA transfer experiments of high-molecular-weight DNA or single oncogenes (e.g., *ras*, *src*, *E1a*) by inducing metastatic competence showed that metastatic ability may have a genetic basis, but they failed to find a metastasis-specific gene and suggested that a multitude of genes contributed to the metastatic behavior of the tumor cells (154,155). In certain recipient cells, the failure to induce metastasis despite the expression of the oncogene may thus reflect some deficiencies in the activation of the necessary downstream genes. More recent approaches comparing gene expression of metastatic and nonmetastatic cells have implicated the importance of loss-of-function genes such as *nm23* (156), *KAI1* (157), and *KiSS-1* (158) in determining the metastatic behavior of tumors. Their forced reexpression suppressed metastasis formation in animal models, and, hence, they were held responsible for the metastatic ability of different tumor types (159). Several of such metastasis suppressor genes have been described (160).

*nm23*, originally found by differential screening of cDNA libraries constructed from metastatic and nonmetastatic clones of the K-1735 murine melanoma, was highly expressed in nonmetastatic cells (156). It was later shown to behave as a metastasis suppressor gene in the same murine melanoma model (161) and in a human breast carcinoma model in nude mice (162). Its forced expression in a human breast cancer cell line reduced the in vitro motility of the cells in response to a number of chemoattractants (163). Expression of the two human homologs (*nm23-H1* and *nm23-H2*, located on chromosome 17) has been shown to correlate inversely with the malignancy (metastatic potential) of some but not all human tumors (164). Unfortunately, the role and the function of *nm23* remain unclear (165). *nm23* displays sequence

homology with nucleoside diphosphate kinase and it shows nucleoside diphosphate kinase activity (166). It may participate in signal transduction involving serine residues autophosphorylation (167). It may also act as a transcription factor, as suggested by the analysis of the motifs present in its sequence (166). It has been suggested that *nm23* may phosphorylate kinase suppressor of *ras* (KSR) protein and by this means impair the KSR scaffold functions for the ERK–mitogen-activated protein kinase pathway. Possibly by altering the docking of proteins or the KSR intracellular localization, it could impact ERK activation. This in turn would lead to a reduced response to the extracellular signaling (168).

*KAIL* (*kang ai*, Chinese for anticancer) is a gene mapping on human chromosome 11p11.2–13. It is expressed in normal human prostate, and its expression was detected at a very low level in five human prostate cancer cell lines derived from metastatic lesions. After transferring the gene into a highly metastatic rat prostate cancer cell line, *KAIL*-expressing cells had reduced metastatic capability while their growth as primary tumor was not affected (157). High *KAIL* expression suppressed metastatic potential of breast cancer cells as well (169). It was reported that the downregulation of *KAIL* is associated with poor prognosis in patients with non-small cell lung cancer (170). Reduction in *KAIL* protein expression observed by IHC analysis of patient specimens was consistent with the progression of the disease (171). Its downregulation did not involve mutations or allelic losses of the gene. *KAIL* protein is located on the plasma membrane of normal prostate epithelial cells and is identical to the glycoprotein encoded by *CD82* gene, a type III integral membrane. Although the precise function of *KAIL* is unknown, its localization and protein sequence suggest that it might be involved in cell–cell and/or cell–ECM interactions. The tetraspanin family of proteins functions as membrane adapters and organizes large oligomeric complexes that may contain other tetraspanin-type proteins (i.e., E-cadherin), integrins, cell adhesion molecules, kinases, GTPases, and phosphatases. *KAIL* complexed with integrins and with epidermal growth factor receptor (EGFR) have been implicated in the metastasis suppression function (172,173).

*KiSS-1* was isolated from malignant melanoma cells by way of a subtraction hybridization strategy in which metastatic C8161 cells and their nonmetastatic derivatives, obtained by the introduction of one intact copy of human chromosome 6, were compared (158). *KiSS-1* gene maps on human chromosome 1q32–q41, suggesting that it might be a downstream effector of gene(s) on chromosome 6. Its function remains unknown, but the deduced amino acid sequence suggests that *KiSS-1* encodes for a protein with a putative SH3 ligand domain. Such a motif is frequently found in proteins involved in signal transduction pathways. Transfection of *KiSS-1* in melanoma and breast cancer cell lines reduced the ability of the transfectants to colonize the lung of nude mice without altering their tumorigenicity (174). The forced expression of *KiSS-1* in a melanoma cell line expressing high levels of MMP-9 markedly decreased MMP-9 expression and activity (175). The gene product and the functional mechanisms are yet to be elucidated. Two groups have suggested that the *KiSS-1* gene encodes for a peptide ligand with agonist activity on orphan G-protein-coupled receptors (176,177).

The usefulness of these metastasis genes in predicting disease outcome is uncertain. It is unlikely that they will represent the universally implicated metastasis-suppressor-gene. By interfering with a single event that is a necessary step of a complex mechanism, the whole biologic program may be blocked. Conversely, it is not possible to evoke the whole process by simulating that single event.

Since each discrete step of the metastatic process appears tightly regulated, metastasis may result from alteration in different genes involved in the most diverse pathways. Moreover, it cannot be excluded that their contribution may occur in a cancer-specific manner. To understand such a complex phenomenon, researchers need to analyze global gene expression and to be able to apply the analysis to a wide range of clinical material.

To this end, microarray technology applied to archival material has allowed the definition of a set of genes able to predict clinical outcome in patients with same-stage disease, namely sporadic lymph node-negative breast cancers. Specifically, the expression behavior of 70 genes in the primary tumor predicted which of the patients would develop distant metastases and which would remain disease free over 5 yr (65/78 correct predictions). The same was also true (17/19 correct predictions) when such a set of genes was evaluated in patients who were not part of the original cohort (178). Together, these findings suggest that even small primary tumors are committed to develop metastasis and indicate that prognosis can be inferred by gene expression profile of the primary tumor.

## Conclusion

Metastasis is a complex process, and its outcome is the result of a number of interactions between cancer cells and the microenvironment. Inhibition of metastases may be achieved by a variety of strategies, including direct impairment of cancer growth and spread, through the blockade of a variety of signal-transduction pathways or indirectly through the control of the host response, such as by affecting angiogenesis and lymphoangiogenesis. The apparent redundancy of the mechanisms involved in the metastatic process may imply the use of more than one therapeutic intervention in blocking metastasis. A better understanding of the molecular basis that regulates metastasis growth is necessary to identify novel and more selective targets for therapy. Furthermore, the individual assessment of specific markers (i.e., expression of adhesion/invasion molecule[s], levels of soluble receptor[s], and alteration of gene[s] associated with the malignant behavior of cancer) may ameliorate prognostic accuracy by helping to define subgroups of patients at risk of developing metastases.

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# 14

## Molecular Pathways of Drug Resistance

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### Introduction

Despite significant advances in the treatment of solid tumors and hematologic malignancies, clinical drug resistance to anticancer therapy is still a frequent problem that often leads to treatment failure in cancer patients. Resistance to chemotherapy can be divided into intrinsic (or *de novo*) and acquired resistance. The former refers to tumors that are insensitive to cytotoxic drugs at diagnosis, such as pancreatic cancer, renal cancer, and malignant melanoma. Acquired drug resistance is common in tumors such as breast cancer, small-cell lung cancer (SCLC), and ovarian cancer that initially are highly responsive to anticancer therapy, but become resistant during the course of the disease. These tumors often develop resistance not only to previously used drugs, but also to other compounds with different structures and mechanisms of action to which they have never been exposed.

Multiple cellular mechanisms have been identified that can contribute to the drug resistance phenotype, including alterations in drug transport systems, resulting in decreased intracellular drug concentration; changes in the activation or inactivation of drugs (metabolic resistance); alterations in drug targets; increased repair of drug-induced damage; alterations in drug-induced apoptosis; and changes in signaling pathways (1–6). In addition, pharmacologic factors, such as inadequate dosing or route of delivery, may play a role in clinical resistance of tumors (Table 1). These mechanisms can develop simultaneously, and multiple factors can contribute to the drug resistance phenotype of tumor cells. Table 2 shows the various resistance mechanisms associated with the anticancer drugs paclitaxel, cisplatin, irinotecan, methotrexate, and doxorubicin. This chapter focuses on the molecular pathways leading to multidrug resistance (MDR) and discusses a series of attempts to manipulate these pathways.

### Alterations in Drug Transport Systems

Reduced intracellular accumulation of drugs is one of the most common mechanisms of MDR. It is usually caused by enhanced drug efflux but can also result from a decreased uptake of the cytotoxic agent caused by defects in the import system (e.g., methotrexate resistance [6]). In addition, intracellular entrapment or redistribution of the drug may decrease the effective intracellular drug concentration. The best charac-

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**Table 1**  
**Mechanisms of Drug Resistance**

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Pharmacologic factors
Inadequate drug dose
Inappropriate infusion rate
Inadequate route of delivery
Drug metabolism and pharmacologic interaction
Cellular factors
Alterations in drug transport systems
Changes in drug activation or detoxification, resulting in metabolic resistance
Alterations in drug targets
Enhanced repair of drug-induced damage
Alterations in drug-induced apoptosis
Changes in signaling pathways

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terized drug exporter in this respect is P-glycoprotein (P-gp), which was first described more than 25 yr ago (7). In recent years, many other transporter proteins involved in MDR have been identified, including the family of MDR-associated proteins (MRPs), the transporter associated with antigen presentation (TAP), the breast cancer resistance protein (BCRP), and the lung resistance protein (LRP). P-gp, MRP, TAP, and BCRP all belong to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of membrane proteins that are able to transport proteins across cellular membranes against a concentration gradient, using energy derived from ATP hydrolysis.

### ***P-Glycoprotein***

P-gp is a 170-kDa plasma membrane protein containing 12 transmembrane domains and 2 ATP-binding sites that can efficiently remove cytotoxic drugs and other substrates from the cell. Besides numerous physiologic products, the substrate list of P-gp contains a broad spectrum of structurally unrelated chemotherapeutic drugs, including anthra-cyclines, *Vinca* alkaloids, taxanes, epipodophyllotoxins, and actinomycin D (Table 3; reviewed in ref. 8). The transmembrane regions of the P-gp bind drug substrates that are probably presented to the transporter directly from the lipid bilayer (9). This binding stimulates the ATPase activity of P-gp, causing a conformational change that releases the substrate either to the extracellular space or to the outer leaflet of the membrane (10). Hydrolysis at the second ATP-binding site seems to be required to reset the transporter, completing one catalytic cycle (11). P-gp is the product of *MDR1*, one of the two members of the *MDR* gene family. Although the product of the other *MDR* gene (*MDR2/3*) has been shown to bind and transport a subset of P-gp substrates to a limited extent (12), only cells expressing MDR1 P-gp display the MDR phenotype (13).

P-gp is expressed in several normal tissues, including the adrenal glands, kidney, liver, pancreatic ducts, gastrointestinal (GI) tract, testes, pregnant uterus, and the capillary epithelium of the blood-brain barrier (14,15). Although its physiologic function is not entirely clear, P-gp appears to be involved in the protection of normal tissues from environmental and endogenous toxins and in secretion and excretion of proteins (15). Thus, P-gp probably evolved to transport a variety of natural toxic products and has coincidentally acquired the ability to transport cytotoxic drugs as well.

**Table 2**  
**Cellular Mechanisms Contributing to Resistance Against Chemotherapeutic Drugs**

	Cisplatin	Irinotecan	Methotrexate	Doxorubicin
P-gp, LRP	MRP2 and 3, LRP	P-gp, BCRP	MRP1, 3, and 4, BCRP	P-gp, MRP1, 2, and 3, LRP
$\beta$ -tubulin mutations	Inactivation by glutathione and metallothionein	Decreased activation by carboxylesterase	Decreased uptake by the RFC	Mutations in topo II
Altered apoptotic response	Enhanced DNA repair Altered apoptotic response	Mutations in topo I Degradation of topo I-DNA complexes	Decreased polyglutamylation Mutated or increased levels of DHFR	Enhanced DNA repair Altered apoptotic response

**Table 3**  
**P-gp Substrates**

Anthracyclines	<i>Vinca</i> alkaloids	Other
Daunorubicin	Vinblastine	Actinomycin D
Doxorubicin	Vincristine	Colchicine
Epirubicin	Vinorelbine	Mitoxantrone
		Mitramycin
Taxanes	Epipodophyllotoxins	Plicamycin
Paclitaxel	Etoposide	
Docetaxel	Teniposide	

Solid tumors originating from tissues that normally express large amounts of P-gp (including colon, kidney, liver, and pancreas) frequently have high expression of the MDR1 transcript and tend to be resistant to many chemotherapeutic agents (14,16). Furthermore, relatively large amounts of MDR1 have been detected in several hematologic malignancies (reviewed in ref. 17). Recent studies in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) showed that MDR1 expression is independently associated with a lower complete remission rate and a higher incidence of refractory disease (18–21). Also in multiple myeloma, MDR1 may play a role in drug resistance (21). In solid tumors, such as sarcomas, SCLC, non–small cell lung cancer (NSCLC), and ovarian cancer, the role of P-gp overexpression in MDR is more controversial (reviewed in ref. 17). A meta-analysis of 31 reports on P-gp expression in breast cancer showed that P-gp expression increased after therapy and was associated with a greater likelihood of treatment failure (22), but there was a considerable interstudy variability. This problem, encountered in many studies, is partly due to the different methods used to detect P-gp expression, and the lack of a quantification standard.

A substantial effort has been made to develop strategies to reverse or circumvent P-gp-mediated MDR. Several agents can partially or completely reverse drug accumulation defects in MDR cells, including calcium channel blockers (e.g., verapamil, nifedipine, bepridil), calmodulin inhibitors (e.g., phenothiazines), immunosuppressive agents (e.g., cyclosporine A) or derivatives (i.e., PSC 883), and many others (reviewed in ref. 23). Clinical trials of MDR modulation are, however, complicated by the presence of multiple mechanisms of drug resistance in human cancers and, until recently, the lack of potent and specific inhibitors of P-gp (reviewed in ref. 24). Furthermore, inhibition of P-gp in normal tissues such as liver and kidney can result in pharmacokinetic interactions due to a decreased clearance, causing increased toxicity (25). Although some trials in hematologic malignancies demonstrated significant clinical benefit from inhibition of P-gp (26), others had to be discontinued because of unacceptable toxicity in the treatment group receiving the P-gp modulator (27). The incidence of toxicity and mortality due to pharmacokinetic interactions seems to depend on the drug administration regimen, the patient population, and the P-gp modulator used. In this regard, new-generation P-gp inhibitors are being developed that have fewer pharmacokinetic interactions, such as biricodar (VX-710) (28) and LY335979 (29). Alternative strategies to specifically inhibit P-gp by down-regulation of its expression are being explored. In this regard, antisense oligodeoxy-

nucleotides that can form complementary double helix structures with their target mRNA and thereby inhibit their translation have been effective in *in vitro* and *in vivo* models (30).

### **MDR-Associated Proteins**

The observation that MDR can be present in tumors and cell lines that do not express P-gp led to the search for other proteins involved in drug transport. In 1992, Cole et al. (31) discovered a second type of drug pump in the SCLC line H69 after stepwise exposure to doxorubicin. This pump, the MRP, is a 190-kDa transmembrane glycoprotein, localized on the plasma membrane and endomembrane structures that belong, like P-gp, to the ABC superfamily (32). Nine members of the MRP family (MRP1–9) have been identified thus far.

MRP1 is generally expressed in normal tissues, where it is probably involved in protection from natural toxins (33). Expression of MRP1 is able to convey an MDR phenotype (34) and may be related to unfavorable treatment outcome (35,36). Despite their common involvement in MDR, function and substrate specificity of P-gp and MRP1 differ. The substrate list of MRP1 includes cytotoxic drugs such as doxorubicin, epirubicin, etoposide, vincristine, and methotrexate, but not mitoxantrone, paclitaxel, or docetaxel (reviewed in ref. 17). In addition, whereas MRP1 transports conjugated organic anions such as leukotriene C4 and drugs conjugated to glutathione, glucuronate, or sulfate, P-gp has a low affinity for such negatively charged compounds (37).

MRP2, also known as the canicular multispecific organic anion transporter (cMOAT), is normally expressed in the liver and, to a lesser extent, in renal proximal tubules. Its physiologic function includes hepatobiliary excretion of conjugated bile salts, and mutations in MRP2 can lead to hyperbilirubinemia II/Dubin–Johnson syndrome (38). Overexpression of this protein is found in many cancer types, such as renal, gastric, breast, lung, ovarian, and colon carcinoma (39). MRP2, like MRP1, confers resistance to a spectrum of anticancer agents including methotrexate, etoposide, doxorubicin, and vincristine. In addition, MRP2 causes resistance to cisplatin, a drug not transported by MRP1 (17).

MRP3, the closest homolog of MRP1, is expressed on the basolateral membrane of liver, gallbladder, colon, pancreas, adrenal gland, and kidney cells (40), and it may play a role in the cholehepatic and enterohepatic circulation of bile and in protection of the biliary tree tissues against toxic bile constituents (41). The protein can induce resistance against etoposide, teniposide, methotrexate, vincristine, doxorubicin, and cisplatin *in vitro* (42,43). Furthermore, upregulation of MRP3 has been found in patients with lung cancer after exposure to platinum compounds (44).

MRP4 and MRP5 do not confer resistance to natural anticancer agents but, instead, are capable of transporting nucleotide analogs and cyclic nucleotides, a feature that suggests their involvement in the regulation of intracellular cyclic nucleotide levels (45,46). MRP4 can confer resistance to antiviral HIV drugs (47) and methotrexate (48,49), whereas both MRP4 and MRP5 overexpression result in moderate resistance against thiopurines (6-mercaptopurine and 6-thioguanine) (45,50).

The *MRP6* gene is highly expressed in liver and kidney (51). Although its physiological function is unknown, *MRP6* gene mutations have been discovered to cause a heritable disease of connective tissues, pseudoxanthoma elasticum (52). It is presently not known if MRP6 or the more recently discovered MRP7, MRP8, and MRP9 play a role in clinical drug resistance (51,53–55).

### ***Transporter Associated With Antigen Processing***

Another member of the ABC transporter superfamily is TAP. TAP is a heterodimer consisting of two subunits, TAP1 and TAP2, that physiologically plays an important role in presentation of major histocompatibility complex (MHC) I-restricted antigens by mediating peptide translocation across the endoplasmatic reticulum membrane (56). Although its role in clinical drug resistance remains to be established, in vitro evidence suggests that enhanced expression of TAP may be related to increased resistance to anticancer agents such as etoposide, vincristine, doxorubicin, and mitoxantrone (57,58).

### ***BCRP/Mitoxantrone Resistance Protein***

The most recently discovered ABC transporter involved in MDR is BCRP, also known as mitoxantrone-resistance protein (MXR). BCRP, a 72-kDa plasma membrane protein containing an ATP-binding domain and six transmembrane domains, confers an MDR phenotype that partially overlaps with that of P-gp-expressing cells (59). Chemotherapeutic drug substrates of BCRP include mitoxantrone, topotecan, irinotecan, methotrexate, and anthracyclines (60,61). The tissue with the highest expression of BCRP is the placenta, but this protein is also expressed in liver, small intestine, colon, breast, and venous and capillary endothelium (62). This expression profile suggests a role of BCRP in protection of the fetus and regulation of transport of chemicals through the epithelium of the GI tract (63). Its role in clinical drug resistance, on the other hand, is not clear. In this regard, conflicting data on the relationship between BCRP expression and increased drug resistance in AML stress the need for further studies (64–66).

### ***Lung Resistance Protein***

Essential for the activity of chemotherapeutic drugs is the intracellular movement toward their site of action, which frequently is the nucleus. Besides ABC transporters, other molecules can divert the drugs from their intracellular target, and thereby confer MDR. In 1993, Scheper et al. (67) identified LRP in a non-P-gp MDR lung cancer cell line. This protein was found to be identical to the major vault protein (68). Vaults are complex ribonucleoprotein particles that are predominantly located in the cytoplasm, but that can also be present in the nuclear membrane and nuclear pore complex (69). The structure and localization of the vaults have led to the hypothesis that vaults mediate the bidirectional transport of a variety of substrates between the nucleus and the cytoplasm (70) and may therefore play a role in drug resistance by regulating the nucleocytoplasmic transport of cytotoxic agents.

LRP is widely distributed in normal tissues and tumors (71), and the phenotype conferred by LRP expression is broad and overlaps with that of P-gp and MRP-expressing cells (72). Overexpression of LRP in human colon, lung, and gastric carcinoma cell lines is associated with resistance to doxorubicin, vincristine, etoposide, cisplatin, gramicidin B, and paclitaxel (73), and expression of LRP correlates with resistance to anthracyclines in childhood ALL (74). Furthermore, expression of functional LRP is associated with poor outcome in adult T-cell leukemia, AML, multiple myeloma, and ovarian carcinoma (75–79). By contrast, no association between LRP and treatment outcome has been seen in breast and lung carcinoma (80,81). To date, no drugs are available that can revert LRP-mediated MDR.

## Metabolic Resistance

Alterations in the activation or detoxification of chemotherapeutic drugs can contribute to clinical resistance of tumors. Irinotecan, for instance, is a prodrug that needs to be converted by a carboxylesterase into its active metabolite, SN-38 (82). Expression levels of this enzyme inversely correlate with irinotecan resistance *in vitro* (83), and adenovirus-mediated transfer of the DNA encoding human carboxylesterase can reverse resistance against this drug in *in vivo* lung cancer models (84). Human carboxylesterase is also present in the liver (85), and the relationship between carboxylesterase levels in tumor tissue and in clinical drug resistance remains controversial (86,87).

Methotrexate is another anticancer drug that needs to be modified for its retention in the cell, which is essential for its cytotoxic function. Methotrexate modification consists of polyglutamylation by the enzyme folylpolyglutamate synthetase (FPGS). Polyglutamylated methotrexate is not recognized by export proteins such as MRP (88) and can accumulate inside the cell (89). Furthermore, while both methotrexate and methotrexate polyglutamates interfere with pyrimidine and thymidylate biosynthesis, only polyglutamylated forms of methotrexate can inhibit DNA synthesis by interference with enzymes involved in purine biosynthesis (reviewed in ref. 4). Decreased polyglutamylation of methotrexate can be caused by alterations in FPGS expression (90) or by enhanced activity of  $\gamma$ -glutamyl hydrolase (GGH), the enzyme that causes breakdown of polyglutamates (91). Preclinical studies indicate that mRNA levels of FPGS and GGH correlate with drug sensitivity (92,93). In patients with ALL and AML, expression of these enzymes may be related to methotrexate response, but further evidence is needed (94–97).

Detoxification of cytotoxic drugs inside the cell is another mechanism of metabolic resistance. An important pathway that leads to inactivation of anticancer drugs is the glutathione/glutathione-S-transferase (GSH/GST) system that conjugates electrophilic metabolites, such as alkylating agents, with the intracellular antioxidant GSH. This reaction, catalyzed by GST, makes these compounds less toxic against cellular targets and more readily excretable by transport systems such as MRP1 and 2. GST forms a multigene family, encoding at least 4 classes of isoenzymes that have partially overlapping substrate specificity (reviewed in ref. 98). Cisplatin; doxorubicin; and most alkylating agents, including melphalan, chlorambucil, and cyclophosphamide, can be inactivated by the GSH/GST system (reviewed in ref. 99). In addition to the GSH/GST system, the metal-binding protein metallothionein can act as an antioxidant and confer resistance to cisplatin, melphalan, and chlorambucil (100,101).

*In vitro* studies have shown that transduction of GSH and GST or metallothionein has chemoprotective effects in human cell lines (102,103). In line with these preclinical data, increased expression of these detoxification systems has been associated with inferior drug response and unfavorable clinical features in cancer patients with several malignancies (104–108). These findings led to the development of inhibitors of detoxification pathways, such as buthionine sulfoximine, an inhibitor of GSH synthesis (109). Although promising results were reported in early clinical trials (110–112), no data are available on the effect of GSH modulation on drug sensitivity in patients with cancer.

## Alterations in Drug Targets

Alterations in the cellular targets of chemotherapeutic drugs may disturb effective drug-target interaction and thus lead to impaired drug response. Defects in  $\beta$ -tubulin,

topoisomerases, dihydrofolate reductase (DHFR), or thymidylate synthase (TS), for example, may render tumor cells resistant to drugs that target these proteins. Paclitaxel is a microtubule-disrupting agent that targets tubulin, a heterodimer consisting of  $\alpha$ - and  $\beta$ -tubulin. Binding of paclitaxel to  $\beta$ -tubulin induces polymerization and bundling of microtubules, which leads to cell-cycle arrest and subsequent cell death (113). Preclinical data indicate that  $\beta$ -tubulin mutations cause impaired polymerization of microtubules, leading to resistance against paclitaxel (114,115). The role of  $\beta$ -tubulin mutations in clinical drug resistance, however, remains, to be established, as clinical studies have shown conflicting results (116,117). Mutations of  $\beta$ -tubulin may have a role in resistance against *Vinca* alkaloids, which exert their cytotoxic function through depolymerization of microtubules (118).

Topoisomerase I and II are critical enzymes involved in DNA replication, transcription, chromosome segregation, and DNA recombination that constitute a target for the topoisomerase inhibitors (irinotecan, topotecan, and etoposide) and anthracyclines. Several alterations affecting topoisomerase I and II, in particular the isoform II $\alpha$ , have been identified that lead to resistance against topoisomerase inhibitors in vitro. These include mutations of topoisomerase I and II $\alpha$  genes, changes in phosphorylation of topoisomerase II $\alpha$ , and reduced expression of topoisomerase II $\alpha$  and II $\beta$  isoforms due to transcriptional downregulation (49,119–124). Clinical studies in patients with lung cancer and multiple myeloma have indicated that high levels of topoisomerase II $\alpha$  and II $\beta$  were predictive for worse clinical outcome (125–127).

The primary cellular target for methotrexate is the enzyme DHFR, which catalyzes the reduction of folate and dihydrofolate to tetrahydrofolate, which is essential for DNA synthesis. Increased amounts of intracellular DHFR, usually due to gene amplification, may cause decreased drug response in human cancer cells (reviewed in ref. 4). As an example, *DHFR* gene amplification was found in 20–30% of patients with relapsed ALL, indicating that this may represent a common mechanism of acquired resistance to methotrexate (95,128). Additionally, DHFR mutations that reduce affinity for methotrexate might result in a decreased drug response in human cancer cells (reviewed in ref. 129).

The cellular target of 5-fluorouracil (5-FU) is the enzyme TS, a key element in the *de novo* synthesis of dTMP from dUMP. Mutations in the *TS* gene and amplification of the gene lead to decreased responsiveness to 5-FU in several models (reviewed in ref. 130). High TS concentrations can be present at diagnosis or induced during treatment, and generally predict resistance to 5-FU, whereas lower concentrations are correlated with better response in colorectal and gastric cancer (131–134). It has been postulated that the response to 5-FU can be more accurately predicted if the expression of TS is analyzed in combination with that of dihydropyrimidine dehydrogenase, the enzyme responsible for degradation of 5-FU (135). Prospective clinical trials are being done that allocate patients for 5-FU or non-5-FU-containing treatment regimens, based on the molecular characteristics of their tumors.

## Enhanced Repair of Drug-Induced DNA Damage

To preserve genome integrity, cells use a precise replication mechanism, and a complex machinery to repair the accidental lesions that occur continuously in DNA. DNA repair processes not only correct accidentally acquired defects, but can also repair damage induced by the action of anticancer drugs, such as platinum compounds and alkylating agents. In this regard, a substantial increase in the repair of DNA lesions



**Table 4**  
**DNA Repair Pathways in Drug Resistance**

Mechanism of repair	Drugs affected	Drugs potentially affected
O <sup>6</sup> -alkylguanine DNA alkyltransferase	Nitrosourea-derivatives, methylating agents	Alkylating agents
Base excision repair	Alkylating agents, ionizing radiation	Anthracyclines
Nucleotide excision repair	Platinum compounds	—
Mismatch repair	—	Methylating agents, doxorubicin, topoisomerase inhibitors, platinum compounds

may conceivably result in reduced chemotherapy-induced cell death and increased drug resistance to DNA-damaging agents (136). Four pathways are involved in the repair of DNA damage induced by anticancer drugs: activity of O<sup>6</sup>-alkylguanine DNA alkyltransferase (AGT), base-excision repair (BER), nucleotide-excision repair (NER), and mismatch repair (MMR) (Table 4) (137). The involvement of each pathway is largely determined by the type of DNA lesion, although the pathways may overlap to some extent.

### **AGT-Mediated DNA Repair**

Chemotherapeutic agents that attack DNA at the O<sup>6</sup> position of guanine include nitrosourea derivatives, such as carmustine and lomustine, which cause interstrand crosslinks, and methylating agents, such as procarbazine, temozolamide, and dacarbazine, which induce DNA-strand breaks (reviewed in ref. 138). Repair of lesions at the O<sup>6</sup>-site of guanine occurs almost entirely through the action of AGT. Using a suicide mechanism, AGT acts as an acceptor protein for the alkyl group, restoring DNA integrity but inactivating itself in the process (reviewed in ref. 139). Tumor expression amounts of AGT vary considerably among individuals, and decreased amounts correlate with better response to chemotherapy in clinical studies in several malignancies (140–143). Inhibition of AGT, achieved by administration of O<sup>6</sup> benzylguanine (BG) (144), potentiates the effects of nitrosoureas in *in vivo* human cancer models (reviewed in ref. 145). In phase 1 studies, BG efficiently decreased AGT activity in peripheral blood mononuclear cells and tumor tissue, without showing significant toxicity (146–148). Phase 2 studies are being done to evaluate the effect of combining BG with nitrosoureas in several malignancies.

### **Base-Excision Repair**

BER is a multistep DNA process that repairs DNA damage induced by ionizing radiation and alkylating agents. The general scheme of BER involves removal of the damaged base by a glycosylase, leading to the formation of an apurinic/apyrimidic (AP) site in the DNA. AP sites can be formed directly through ionizing radiation and oxidative agents such as anthracyclines and can be processed by two branches of the BER pathways that both use DNA polymerases and ligases to fill the gap. The BER

short-patch pathway involves the repair of one isolated AP site, whereas the more complex long-patch pathway can replace up to 13 nucleotides surrounding the AP site (reviewed in ref. 149).

Because many proteins are involved in the BER process and several partially overlapping pathways coexist, the clinical relevance of this repair mechanism is difficult to establish. Gene transduction studies showed that several BER enzymes such as the glycosylase MPG/AAG does not increase or only modestly increases resistance against alkylating agents (reviewed in ref. 137). Recent studies on the glycosylases Fpg and  $\alpha$ -hOgg1 suggested, however, that mammalian cells can be protected from chemotherapeutic agents such as thiotepa, BCNU, and mafosfamide by the BER pathway (150,151). In line with these findings, methoxyamine, which disrupts the DNA repair process by preventing AP endonuclease cleavage, can enhance the cytotoxicity of methylating agents (152).

### **Nucleotide-Excision Repair**

The nucleotide excision pathway is a complex DNA repair mechanism that can remove bulky DNA lesions up to 27–29 nucleotides. Rather than searching for specific base changes, NER scans the DNA for distortions in the double helix that interfere with base pairing and generally obstruct normal transcription and replication. These changes can be caused by ultraviolet (UV) light and platinum compounds, among others. Two NER subpathways exist with partially distinct substrate specificity: global genome NER surveys the entire genome for distorting injury, and transcription-coupled NER focuses on damage in the transcribed DNA strands of active genes (reviewed in ref. 153). Repair initiated by NER is a sequential, multistep process, that involves recognition of the DNA damage, opening of a region around the lesion, dual incision, and repair synthesis (154).

Platinum compounds are the prototype of drugs studied in relation to the role of the NER pathway in drug resistance, because of the bulky DNA lesions they induce. In fact, NER, in particular transcription-coupled NER (155), is assumed to be one of the most important determinants of cisplatin resistance (2). Among the many components of the NER complex, the excision repair cross-complementary group 1 (*ERCC-1*) gene is crucial with respect to drug resistance (156). In vitro studies show that increased amounts of *ERCC-1* mRNA correlate with resistance against cisplatin (157,158). Alternatively, decreasing *ERCC-1* expression by using antisense RNA abrogates gemcitabine-mediated synergism with cisplatin in human colon cancer cells (159). In cancer patients, amounts of *ERCC-1* mRNA are predictive for response and survival after cisplatin treatment for gastric, colon, and NSCLC (134,160–162). In preclinical investigations, the novel inhibitor of the NER pathway F11782 increased the effect of cisplatin, providing a basis for its evaluation in clinical trials in combination with DNA crosslinking agents (163).

### **Mismatch Repair**

Mismatches that breach the Watson–Crick base-pairing rules (A and G pair with T and C, respectively) can occur as a consequence of incorrect nucleotide incorporation during DNA replication. This type of defect is corrected by the MMR pathway, which plays a critical role in guarding the integrity of the genome in virtually all species from bacteria to humans (reviewed in ref. 164). Defects in the human MMR system are responsible for predisposition to hereditary nonpolyposis colorectal cancer (165).

**Table 5**  
**Important Pro- and Antiapoptotic Proteins**  
**Involved in Drug Resistance**

Proapoptotic proteins	Antiapoptotic proteins
Bcl-2 family members	Bcl-2 family members
Bax	Bcl-2
Bcl-x <sub>S</sub>	Bcl-x <sub>L</sub>
Bak	Bcl-w
Bad	Mcl-1
Bid	A1
	Bag-1
p53	IAP proteins
c-myc	cIAP-1
	cIAP-2
	XIAP
	Survivin

In contrast to other DNA repair pathways, it is deficiency in MMR functions instead of increased activity that causes resistance to various chemotherapeutic agents in vitro, including methylating agents (temozolomide and procarbazine), doxorubicin, etoposide, topotecan, and platinum compounds (166–169). As an explanation for this somewhat surprising observation, it has been proposed that the MMR system may trigger programmed cell death (apoptosis) when confronted with the extent of DNA damage induced by chemotherapeutic drugs. Thus, a reduced activity of the MMR system would lead to fewer apoptotic events, causing cellular protection against the cytotoxic effects of certain chemotherapeutic drugs (reviewed in ref. 170). To date, the few data that are available on the clinical relevance of defective MMR pathways do not indicate a relationship between expression of *MMR* genes and drug sensitivity, but further evidence is needed (171,172).

**Defective Apoptotic Response**

In the past 10 yr, cumulative evidence has indicated that antineoplastic agents exert at least part of their cytotoxic effects by induction of a common death pathway, known as apoptosis. This process can be initiated by intrinsic and extrinsic pathways, which both lead to a cascade-like activation of caspases, a family of proteases that are the final executioners of apoptosis, resulting in degradation of cellular proteins and disassembly of the cell (173). The apoptotic cell death program is tightly regulated by various proteins and is responsible for normal tissue homeostasis, which is achieved through a balance between cell death and cell proliferation. As a consequence, deregulation of the apoptotic pathways favors carcinogenesis by providing tumor cells with a survival advantage compared with their normal counterparts (174), and novel therapeutic approaches are being explored that specifically trigger apoptosis in cancer cells (175). In addition, because many anticancer agents exert their effect at least partially through activation of the apoptotic cascade (176), alterations in apoptosis can lead to broad-spectrum drug resistance, irrespective of the intracellular target. Among the genes implicated in the regulation of apoptosis, *p53*, *Bcl-2* family members, *c-myc* and the inhibitors of apoptosis proteins (Table 5) have been studied with respect to their relation with sensitivity to chemotherapy and are discussed here.

## p53

The tumor suppressor gene (TSG) *p53* encodes a 53-kDa nuclear phosphoprotein that functions as a transcription factor and plays a crucial role in cellular processes such as cell-cycle regulation at the G1/S-phase and the G2/M-phase checkpoints, induction of apoptosis, and maintenance of genomic stability (177). Because each of these processes is indispensable for cellular homeostasis, alterations in the *p53* gene may play a central role in the multistep carcinogenesis process and may affect the prognosis and response to therapy of a variety of tumors. Normally, cellular p53 is kept at a low concentration by its relatively short half-life, but it can be induced by a variety of stimuli including ionizing radiation, hypoxia, and UV light, and by DNA damage caused by cytotoxic agents. On activation of p53, either cell-cycle arrest allowing for DNA repair processes or the triggering of apoptosis may occur, depending on the type and severity of damage and the presence or absence of survival factors (reviewed in ref. 178). Although the precise interactions remain to be elucidated, genes involved in DNA repair and p53 appear to have a close collaboration (reviewed in ref. 179). The pivotal functions of p53 explain why selection for p53 mutations occurs in tumors, as p53 triggers apoptosis in cells with activated oncogenes (177).

*p53* gene mutations are common in human cancer and are mostly located in the highly conserved core domain of the *p53* gene (180). Besides mutational inactivation, p53 can become inactive through interactions with its physiologic counterpart mdm2 (181) or with viral proteins such as E6 (182). The dual role of p53 as a mediator of apoptosis and inducer of cell-cycle arrest implies that *p53* mutations could increase or decrease chemosensitivity to anticancer agents. Current preclinical and clinical data provide evidence for both scenarios. Whereas initial in vitro and in vivo evidence pointed to an increase in chemoresistance caused by loss of p53 function (183–186), it was also observed that p53 inactivation could correlate with increased chemosensitivity in some systems (187,188). Thus, the relationship between p53 status and chemosensitivity in preclinical models is complex and is presumably dependent on several factors, including the specific cytotoxic stimuli, tissue-specific differences, and the specific cellular context.

In addition to the contradictory preclinical data, clinical studies are inconclusive with respect to the effect of p53 status on tumor response in cancer patients. Some studies show a correlation between mutant *p53* and decreased chemosensitivity, whereas in others p53 was not predictive for treatment outcome (189). Published studies have produced contradictory findings on the impact of mutant p53 on drug resistance in the most common solid tumors (190–198). Some pitfalls associated with the methodology used in the clinical studies, such as low sample size, detection method (immunohistochemical staining vs single-strand conformation polymorphism and mutational analysis), use of frozen vs fixed tumor tissue, and cutoff points for positivity, may contribute to these conflicting results (199). In summary, p53 is crucial for regulation of the cell cycle and induction of apoptosis and is likely involved in cell death induced by antineoplastic therapies, but its precise role in chemosensitivity cannot yet be defined and needs further investigation in large, carefully designed prospective studies.

The pivotal role of p53 in cell-cycle control and apoptosis and its function as a TSG has led to attempts to modulate p53 in human malignancies. *p53* gene transfer mediated by retroviral or adenoviral expression vectors restored drug and radiation sensitivity in vitro (200) and showed evidence of clinical activity when given in combination

with cisplatin (201). *p53* gene transfer did not provide, however, any additional benefit in patients receiving an effective first-line chemotherapy for advanced NSCLC in another trial (202).

### ***Bcl-2 Family of Proteins***

The *Bcl-2* gene was originally described at the chromosomal breakpoint of t(14; 18)-bearing B-cell lymphomas (203). The first evidence for the biologic function of this protein was obtained in in vitro experiments in which enforced expression of Bcl-2 led to long-term survival of hematopoietic cell lines after growth factor deprivation (204). Other experiments showed that Bcl-2 prevents apoptosis and is indispensable in maintaining the viability of hematopoietic stem cells (205). Thus far, several Bcl-2 family members have been identified that are either proapoptotic or antiapoptotic and differ in their expression patterns and structural features (206). A rheostat model has been proposed in which the ratio of death antagonists (Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, Mcl-1, A1, Bag-1) to death agonists (Bax, Bad, Bid, Bcl-x<sub>S</sub>, Bak) determines the fate of cells after an apoptotic signal (207). The determinants of the ratio of Bcl-2 family members are poorly understood, but they appear to be controlled at both transcriptional (e.g., p53-dependent transactivation of Bax) (208) and posttranslational (e.g., phosphorylation of Bcl-2 induced by antimitotic agents) levels (209).

The role of Bcl-2 family members in chemoresistance has been investigated extensively in in vitro systems, animal models, and clinical studies. Preclinical data showed that Bcl-2 family alterations contribute to resistance to various chemotherapeutic agents, including cytarabine, methotrexate, doxorubicin, paclitaxel, etoposide, and cisplatin (210). Several studies in AML have indicated that high Bcl-2 expression and a high Bcl-2/Bax ratio were independent prognostic factors for unfavorable clinical outcome (211–213), whereas no relationship between Bcl-2 or Bax expression and drug resistance could be seen in ALL (214,215). Additionally, some investigators found that low amounts of Bax, Bcl-2 overexpression, and Bcl-2 rearrangement correlated with poor response in lymphomas (216–218), whereas others could not confirm such a relationship (219–221). The data in solid tumors are even more contradictory and do not provide clear evidence to draw a conclusion on the role of Bcl-2 family members in drug resistance (222–227). Apart from methodologic issues, these paradoxical data on Bcl-2 may be due to inactivation, inhibition, or loss of the downstream apoptotic machinery in apoptosis-resistant (solid) tumor cells. Because aggressive cancers may not further depend on Bcl-2 after losing functional apoptotic pathways, this protein may become irrelevant for cell death, and amounts of Bcl-2 may decline in such cells. By contrast, in hematopoietic and leukemic cells, which are prototypes for apoptosis-prone cells, overexpression of Bcl-2 is needed to prevent cell death and may predict tumor response to cytotoxic agents (228).

Research has focused on affecting the Bcl-2 status of cancer cells in an attempt to restore the natural occurrence of apoptosis and to enhance the effects of chemotherapy. For this purpose, antisense oligonucleotides have been developed that have been shown to facilitate tumor cell apoptosis and to sensitize tumor cells to cytotoxic treatments in preclinical models (reviewed in ref. 229). Phase 1 trials showed that Bcl-2 antisense therapy is safe and well tolerated when given in combination with chemotherapy (230,231), but no studies are as yet available that address the effect on chemosensitivity in human tumors of Bcl-2 antisense therapy.

## **c-myc**

The oncogene *c-myc* is a transcriptional regulator that plays an important role in growth control and cell-cycle progression. Its ability to modulate the transcription of a wide range of target genes gives c-myc the capacity to affect multiple pathways involved in proliferation, apoptosis, growth, and transformation. By generating an excessive proliferating signal, c-myc produces a paradoxical effect that leads to apoptosis and may influence the response to chemotherapy (reviewed in ref. 232). In vitro studies confirm that amplification of c-myc is associated with an apoptosis-prone phenotype of human colon carcinoma cells (233,234). In line with these preclinical data, the few clinical studies that have investigated the relationship between c-myc expression and chemosensitivity indicate that low-level amplification of c-myc is correlated with better response to chemotherapy (233,235), especially in patients with wild-type tumoral p53 (236).

## **Inhibitors of Apoptosis Proteins**

Several members of the inhibitor of apoptosis (IAP) family have been shown to inhibit cell death mainly through interaction with caspases. IAPs were first discovered in baculoviruses but are highly conserved throughout mammals. They comprise a growing family consisting of NIAP; Apollon; c-IAP1; c-IAP-2; XIAP; survivin (reviewed in ref. 237), and the recently discovered TUCAN (238), Livin (239), and Aven (240), which may be involved in oncogenesis and tumor development (241). In vitro, overexpression of IAP has been shown to protect tumor cell lines from apoptosis induced by different anticancer agents (reviewed in ref. 237). In the clinical setting, however, the relationship between IAP expression and chemosensitivity may be more complex than anticipated by in vitro data. In 55 patients with NSCLC, expression of c-IAP1, c-IAP2, and XIAP did not correlate with response to chemotherapy (242). Expression of survivin is commonly found in human cancers and has frequently been described to be an unfavorable prognostic factor, although it was often unrelated to tumor response (243–247).

## **Alterations in Signaling Pathways**

Cellular processes such as cell proliferation and differentiation are controlled by various signal transduction pathways, and altered signaling through these pathways is involved in oncogenesis and tumor development. The development of biologic response modifiers, which target abnormal signaling pathways in human malignancies, has led to the recognition of alternative resistance mechanisms that stem from alterations in signaling pathways. Her2, a member of the erbB receptor tyrosine kinase family that is frequently overexpressed in breast cancer, can cause enhanced DNA repair, defective cell-cycle checkpoints, and altered apoptotic responses, resulting in resistance against DNA-damaging agents and antimitotic drugs in vitro (248,249). Not all in vivo studies could confirm these findings, and some controversy remains with respect to the effect of Her2 expression on chemosensitivity in human cancer (reviewed in ref. 250). Nevertheless, combination studies of the recombinant human anti-Her2 antibody trastuzumab (Herceptin) with chemotherapy in breast cancer demonstrated that response rates are higher than expected for either single agent alone, indicating that overexpression of Her2 may be a clinically relevant mechanism of drug resistance that may be partially overcome by trastuzumab (251,252). Although trastuzumab has some activity in

patients with Her-2-positive breast cancer, resistance to this agent is present in most patients (253). The mechanisms of trastuzumab resistance are not well understood.

Imatinib mesylate is a tyrosine kinase inhibitor that inhibits the constitutively active fusion protein Bcr-Abl arising from the Philadelphia chromosome, and the oncogenic protein *c-kit*. Both *c-kit* and Bcr-Abl are members of the tyrosine kinase family and promote cancer cell growth in gastrointestinal stromal tumor (GIST) and chronic myelogenous leukemia (CML), respectively. In both diseases, imatinib mesylate is highly active and induces responses in up to 95% of patients in chronic-phase CML and in approx 40% of patients with GIST (254). Response rates are much lower in blast-phase CML, and relapses occur despite continuous therapy (reviewed in ref. 255), suggesting that mechanisms of resistance against this agent either preexist or can develop under treatment. The relative resistance of blast-phase CML is consistent with the hypothesis that secondary mutations, and not Bcr-Abl itself, are the driving force of cell growth, as blast cells are genetically more unstable and have additional molecular changes than cells in chronic-phase CML (256). In addition, 11 patients with relapsing CML with acquired drug resistance to imatinib mesylate was found to be due to *Bcr-Abl* gene amplification, or mutations in the *Bcr-Abl* gene (257). Several point mutations in the region of the tyrosine kinase domain of Bcr-Abl have been described to result in decreased binding of imatinib mesylate leading to clinical drug resistance (258–261).

## Conclusion

Despite its achievements, the effectiveness of chemotherapy is still hampered by the development of drug resistance. To prevent the occurrence of broad-spectrum drug resistance, combination regimens have been designed. In addition, attempts have been made to modulate resistance pathways by, e.g., inhibiting P-gp, decreasing the cellular drug detoxification potential, and affecting p53 or Bcl-2 status. These efforts, however, are hampered by the coexistence of multiple, partially unidentified resistance mechanisms in human cancer. Recently, progress has been made in the field of gene sequencing and microarray technologies and these techniques could be helpful identifying genetic abnormalities in individual human tumors that could be relevant for treatment response. Genetic profiling may enable clinicians to develop individualized treatment regimens, based on the molecular characteristics of the tumor that induce higher response rates than standard therapies. It is hoped that further studies on determinants of response and resistance of human tumors will lead to the development of more efficacious treatment regimens.

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# 15

## Genomic Instability, DNA Repair Pathways, and Cancer

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Gabriel Capellá and Miguel Angel Peinado

### Introduction

Genetic information, in the form of DNA, must be protected for expression within the cell and transfer between generations. While a number of mechanisms have been developed to allow for genetic diversity, multiple and redundant mechanisms work to avoid errors during DNA synthesis before cell duplication and/or to avoid the impact of mutagens from endogenous or exogenous sources. The DNA sequence can be changed as the result of copying errors introduced by DNA polymerases during replication and by environmental agents such as mutagenic chemicals and certain types of radiation. If DNA sequence changes, whatever their cause, are left uncorrected, both growing and nongrowing somatic cells could accumulate so many mutations that they could no longer function. Thus, the correction of DNA sequence errors in all types of cells is important for survival (1).

### Genetic Basis of Cancer

Cancer cells share a number of characteristics, including self-dependence on positive regulatory signals, a lack of response to growth inhibitory signals, limitless proliferation, resistance to apoptosis, the capability to obtain nutrients and oxygen by angiogenesis, and the ability to invade and establish distal metastasis (2). It is currently accepted that, underlying acquisition of the malignant phenotype, cells accumulate mutations in two classes of genes—protooncogenes and tumor suppressor genes (TSGs)—through a multistage process (3). Most of the mutations that contribute to the development and behavior of cancer cells are somatic (i.e., are generated during tumor development and are present only in the neoplastic cells of the patient). Only a small fraction of all mutations in cancer cells are present in the germline predisposing to cancer.

TSGs have been defined as those genes inactivated by germline or somatic mutations in cancer (4). Two types of suppressors can be envisioned: gatekeepers and caretakers (3,5). Gatekeepers act directly to regulate cell proliferation and/or apoptosis and are rate limiting for tumorigenesis. The *APC* gene in human colorectal tumorigenesis is an example (6). Caretakers, also affected by inactivating mutations in cancer, do not directly regulate proliferation. When mutated, they lead to accelerated conversion of a

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normal cell to a neoplastic cell through an increased frequency of mutations in other cellular genes, particularly genes that are rate determining in tumor development (5).

It must be emphasized that distinguishing between what constitutes a growth-regulating TSG vs a DNA-repair TSG may be difficult. Some TSGs, including perhaps *BRCA1* and/or *BRCA2*, may ultimately be found to have functions in both growth control and DNA repair. Nevertheless, present data, suggest that loss-of-function mutations in both alleles of certain DNA repair pathway genes, such as the DNA mismatch repair (MMR) genes, probably do not directly alter cell growth.

### **Genetic Instability is a Key Feature in Tumor Progression**

Tumor development and progression are characterized by the accumulation of genetic alterations. Thus, human cancer can be envisioned as a disease of underlying genetic instability. A dynamic tumor model in which genomic instability would play a principal role in the generation of heterogeneity in cancer has been proposed (7). Genetic instability may exist within tumors, and genetic changes, a consequence of this instability, are responsible for cell heterogeneity. Biologic and genetic tumor cell heterogeneity, a hallmark of most human cancers, provides a framework for interpreting tumor progression as an evolving process. In this model, variant cells emerge throughout the evolution of the tumor, and cell subclones are subsequently selected according to their biologic behavior (i.e., metastatic ability, drug resistance). Genetic drift and its modulating factors would shape tumor progression.

### **Mutator Phenotype Hypothesis**

The most direct consequence of genomic instability is genomic damage. Chromosomal analysis of tumor cells, as assessed by conventional cytogenetics or comparative genomic hybridization or flow cytometry, has produced a significant amount of amplifications, deletions, and change in chromosome numbers that has been proposed as the initiating event in carcinogenesis (8). Allelic imbalance analysis at the subchromosomal levels, using either microsatellite markers or other DNA fingerprinting methods, has also shown widespread changes in copy number in alterations present in tumor cells (9,10). To account for the disparity between the rarity of mutations in normal cells and the large number of alterations detected in tumor cells, Loeb (11–13) proposed the mutator phenotype hypothesis. He argued that an early step in tumor progression is the expression of a mutator phenotype resulting from mutations in genes that normally guarantee the fidelity of DNA synthesis or the adequacy of DNA repair. Mutations in these genetic stability genes could produce additional mutations throughout the genome, affecting both genes controlling growth and genes with other roles in the maintenance of DNA instability.

### **Origin of DNA Damage**

The relevance of DNA damage and repair to carcinogenesis became evident when it was recognized that agents causing cancer—carcinogens—are also mutagens that change the DNA sequence. All effects of carcinogenic chemicals, ultraviolet (UV) radiation, or ionizing radiation on tumor production can be accounted for by the DNA damage that they cause and by the errors introduced into DNA during the cells' efforts to repair the damage. The contribution of endogenous DNA damage to cancer is being increasingly recognized (14). Undue DNA replication; spontaneous loss of bases due to spontaneous disintegration of chemical bonds; and DNA damage secondary to

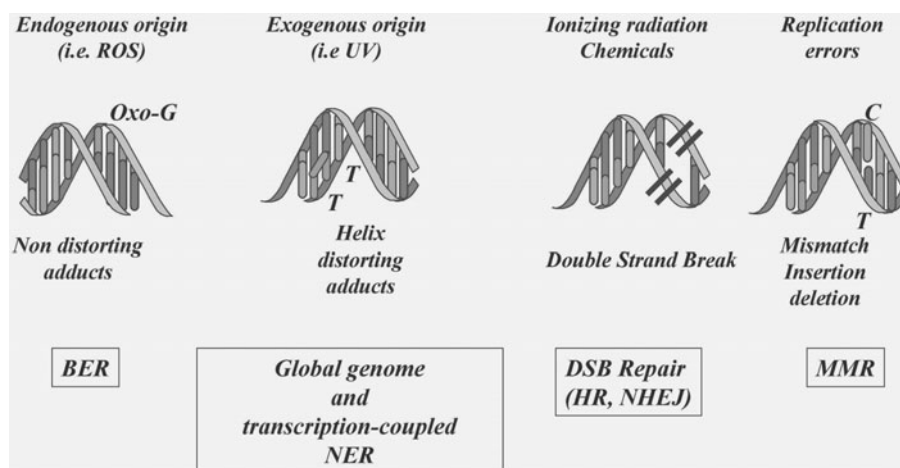


Fig. 1. Main types of DNA repair pathways depicting origin of the damage, type of damage produced, and repair pathway involved.

endogenous reactants such as alkyl groups, metal cations, and reactive oxygen species (ROS) are also important sources of DNA damage. When cells fail to adequately repair the acquired damage, carcinogenesis may occur. A link between carcinogenesis and failure of DNA repair was suggested when humans with inherited genetic defects in certain repair systems were shown to have an enormously increased probability of developing certain cancers such as xeroderma pigmentosum (XP).

## Responses to DNA Damage

DNA damage elicits a number of responses including

- Sensing and recognizing DNA damage by activation of cell-cycle checkpoints, pauses that permit assessment and completion of DNA processing, either DNA damage repair or processing of DNA intermediates
- Upregulation of a large number of genes
- Apoptosis when the cell is unable to repair the damage sustained
- Damage tolerance associated with the newly documented error-prone polymerases
- Multiple distinct DNA repair responses (15)

These responses can be classified into at least four main categories, which are partially overlapping and deeply interrelated. In this section, we briefly describe what is known about them and discuss their putative contribution to cancer development.

### Base-Excision Repair

Small chemical adducts (methylated or oxygenated bases), usually of endogenous origin, can be repaired by base-excision repair (BER), and this pathway has an important role in repair of ROS-induced damage (16) (Fig. 1). After recognition of the adduct, DNA glycosylases (up to eight enzymes have been identified in humans) excise the modified base leaving an apurinic/apyrimidic site. Then a complex composed of APE1 (apurinic endonuclease), DNA polymerase  $\beta$ , DNA ligase 3, and XRCC1 targets the site to produce a short patch of repaired DNA (15). Alternatively, in a few cases, a repair involving a long patch of DNA can occur. No human disorders caused by inherited BER deficiencies have been described.

### **Nucleotide-Excision Repair**

Nucleotide-excision repair (NER) removes >1 base in response to adducts resulting in helix-distorting conformation that impairs transcription and normal replication (Fig. 1). NER is the most versatile system in terms of lesion recognition. Two NER subpathways have been proposed—global genome NER and transcription-coupled repair—that focus on damage that blocks elongating RNA polymerases (15,17). Although it is controversial, the latter has been considered a distinct pathway of repair, a position supported by findings in Cockayne's syndrome (15).

The global genome NER process includes

- Damage recognition (involving XPA, XPC, and RPA proteins and the TFIIH complex of transcription machinery)
- Incision of the DNA strand (performed by XPG and XPF complexed with ERCC1)
- Removal of the adduct that is embedded in a DNA 25mer

Then resynthesis is completed by polymerases  $\delta$  or  $\epsilon$  interacting with proliferating cell nuclear antigen followed by a final ligation step. Transcription-coupled NER machinery is quite different. Transcription arrested by RNA polymerase II is believed to recruit a multimeric complex composed of XP, MSH, CSA, and CSB proteins. Then NER or BER machinery is recruited according to the type of lesion encountered during transcription (17). The presence in this complex of proteins involved in other types of repair reinforces the notion of overlapping between the distinct DNA repair pathways.

At least three hereditary syndromes (XP, Cockayne's syndrome, and trichothiodystrophy) are associated with defects in NER, all of them sharing the trait of exquisite sun sensitivity (Table 1). The prototype, XP, an autosomal recessive disease arising in one of seven genes (XPA–XPG), increases the risk of developing skin cancer. Cells of affected patients are unable to repair UV-induced DNA damage or to remove bulky chemical substituents on DNA bases. The complexity of mammalian excision-repair systems is shown by the fact that mutations in at least seven genes lead to XP lesions, all of them displaying overlapping phenotypes (15). The molecular basis of a variant form of the disease, associated with functional NER, has been elucidated. In these cases, the gene responsible for the disease is DNA polymerase  $\eta$ . This polymerase is highly error prone and can process damaged DNA at an acceptable error rate. Although in critical situations it may be a survival tool for a cell, the price for this trait is DNA error accumulation (18–20).

The relationship between DNA repair and cancer is not straightforward, however. The remaining two hereditary syndromes associated with inborn defects in other proteins involved in NER (i.e., Cockayne's syndrome, characterized by exquisite photosensitivity and early aging, is secondary to mutations in *CSA* or *CSB* genes; and trichothiodystrophy, secondary to alterations in member of the XP proteins) do not increase cancer risk (Table 1).

### **Double-Strand Break Repair**

Double-strand breaks (DSBs) are potent inducers of mutations and cell death (21). DSBs arise from ionizing radiation, X-rays, free radicals, or chemicals (Fig. 1). They can also occur during replication of a single-strand break or in collapsed replication forks. Sensing DSBs is a crucial component of a DNA repair pathway (22). The product of the *ATM* gene, which is mutated in the cancer-prone disorder ataxia telangiect-

sia (AT), appears to be a major sensor of DSBs present after exposure to ionizing radiation. After activation, ATM phosphorylates a number of different substrates including p53, mdm2, Chk2, Nbs1, Brca1, and Smc1, all believed to be involved in downstream signaling of the DNA damage response. A homolog of ATM, ATR (AT and Rad53-related), appears to be involved in the response to UV-induced damage, playing a role in the recognition and repair of DNA-repair complexes that have stalled at sites of DNA damage (5).

DSBs are more of a problem than other types of damage, i.e., DNA adducts or abasic sites, because the cell must “know” which ends belong together without the other copy of the DNA molecule. Two repair pathways, homologous recombination and end joining (and maybe other backup systems), are used (22,23). When a second identical DNA copy is available, usually during G2/M-phase when sister chromatids are available, homologous recombination seems to be preferred because it is an error-free system. Otherwise, cells rely on nonhomologous end joining (NHEJ) that is more error prone.

Main components of the homologous recombination system include the multiprotein RAD50/MRE11/NBS1 nuclease complex (24) that resects flush DSBs to generate single-strand DNA tracts amenable for repair (21). This complex is likely to play other roles such as DNA damage signaling (24). Then a RAD51 complex that includes XRCC2, XRCC3, BRCA1, and BRCA2 forms a nucleoprotein filament that searches for the homologous duplex. DNA synthesis is followed by ligase action after gap filling and subsequent resolution of Holliday junctions by resolvases such as members of the RecQ family of DNA helicases (1).

End joining does not rely on an additional DNA copy. After recognition of the DSB by end-binding proteins KU70p, Ku80p ligation is performed by the complex DNA-PKligase IV and XRCC4 protein (14). Sometimes, this process is associated with gain or loss of a few nucleotides, and DNA polymerases may be used. The role of the MRE11 nuclease complex in this process has not been clarified.

The relationship between alterations in components of the DSB repair pathway and cancer is more evident (Table 1). AT is a cancer-prone, X-ray-sensitive syndrome secondary to mutations in the *ATM* gene. Affected individuals develop progressive cerebellar ataxia and have a 30–40% lifetime risk of lymphoid malignancies (1). AT cells have spontaneous chromosomal instability and fail to suppress DNA synthesis in response to ionizing radiation. AT heterozygous variant carriers are present in approx 1% of the population, and the genotype may be responsible for a large amount of background genetic influence on the incidence of cancer in the population (25). Mutations in the *MRE11* multiprotein nuclease complex also result in an AT-like syndrome characterized by milder AT symptoms and lack of telangiectasias; however, its cancer risk is unknown (24). Inborn defects in the NBS1 component of the nuclease are associated with Nijmegen breakage syndrome, characterized by microcephaly, mental retardation, and predisposition to lymphoma.

While these syndromes are rare, other proteins involved in the homologous recombination pathway, BRCA1 and BRCA2, are associated with a more common cancer-prone disorder—familial breast cancer (Table 1). Approximately 15–20% of the observed risk in such families can be attributed to mutations in *BRCA1* or *BRCA2* genes. When families have more than six affected relatives, *BRCA* mutations may explain 80% of the risk (26). Loss of heterozygosity for the genes is frequently found in breast and ovarian tumors arising in affected relatives, supporting the role as TSG.



**Table 1**  
**Human Hereditary Diseases Associated with DNA Repair Defects and Cancer Predisposition**

Protein	Function	Human disease	Cancer predisposition
Global genome NER XPA, XPC XPB, XPD XPE XPF, XPG XPB, XPD	Damage recognition/binding Helicase in transcription complex DNA Endonuclease single-stranded DNA Helicase in transcription complex	Xeroderma pigmentosum	Skin carcinomas and melanomas
Polymerase $\eta$	Translesion synthesis at adduct	Trichothiodystrophy Xeroderma pigmentosum Variant form	None Skin carcinomas and melanomas
Transcription-coupled NER CSA CSB	Transcriptional activator/transcription coupling	Cockayne's syndrome	None; early aging
DSB repair ATM	Protein kinase; related to PI3K DNA damage signal transduction	Ataxia telangiectasia	Lymphomas
MRE11	Nuclease belonging to multiprotein MRE11 complex	Ataxia telangiectasia-like syndrome	Unknown

NSB1	{	Protein of unknown function belonging to multiprotein MRE11 complex	{	Nijmegen breakage syndrome	Lymphoma
BRCA1	{	Sensing/signaling Participates in transcription-coupled NER	{	Hereditary breast cancer	Breast, ovary
BRCA2	{	Controls RAD51 recombinase activity Participates in transcription-coupled NER	{	Hereditary breast cancer	Breast, ovary
BLM WRN RECQL4	{	Helicase of RecQ family involved in resolving Holliday functions		Bloom's syndrome Werner's syndrome Rothmund-Thomson syndrome	Whole spectrum Sarcoma Sarcoma, osteosarcoma
MMR					
HMSH2 hMSH6	{	Mismatch recognition Transcription-coupled NER DSB repair	{	HNPCC	Colon, endometrial, small bowel, urinary tract
hMLH1	{	DNA excision Transcription-coupled NER DSB repair			
DNA damage signaling P53 CHK2		Transcription factor. Serine/threonine kinases	{	Li-Fraumeni syndrome	Breast, soft-tissue sarcomas, brain tumors, osteosarcoma, leukemia, adrenocortical carcinoma
PI3K, phosphatidylinositol 3' kinase					

Targeted disruption of these genes in mouse models leads to increased sensitivity to hydrogen peroxide or ionizing radiation. BRCA2 controls RAD51 recombinase activity while the role of BRCA1 is apparently a more general one, interacting with both sensing/signaling functions and effector components (i.e., RAD51, RAD50, Rb, and p53 proteins among others) of the DSB pathway (5). The relevance of BRCA in maintenance of genomic instability is reinforced by the fact that its role is not restricted to the DSB and is also involved in transcription-coupled repair of oxidative damage.

Inborn defects in genes of the RecQ family of DNA helicases (i.e., BLM, WRN, and RecQL4) are also responsible for some rare cancer-prone syndromes (27) (Table 1). This class of enzymes can move along a DNA duplex using the energy of adenosine triphosphate hydrolysis to separate the strands and are believed to play a role in solving Holliday junctions, the last step in the DSB repair pathway. Three hereditary syndromes have been associated with defects in these enzymes. Bloom's syndrome is characterized by proportional dwarfism, birdlike facies, photosensitive rash, reduced fertility, and premature aging. Affected individuals are at increased risk of the whole spectrum of malignant disease, and mutations in the *BLM* gene are the underlying cause of the disease (28). Werner's syndrome and Rothmund–Thomson syndrome, secondary to *WRN* and *RECQL4* gene mutations, respectively, share several features with Bloom's syndrome (5,29). The latter two syndromes have increased risk of sarcoma development. The characteristic cellular feature of Bloom's syndrome is genetic instability associated with an increased rate of sister chromatid exchanges. While both BLM and WRN proteins may play a role in translocation of Holliday junctions, they also interact with distinct proteins involved in different steps of several DNA repair pathways, leaving its role partially unknown (30).

Another relationship between DSB repair and cancer has been found through the study of telomere functions. Telomeres prevent the ends of eukaryotic chromosomes from being recognized as DNA breaks. Thus, a connection between telomeres and DSB repair machinery could be envisioned. The Ku86 protein, central to the NHEJ repair, mediates both end-to-end chromosome fusions and apoptosis triggered by short telomeres (31,32); however, the interplay between telomeres and DNA repair may be even more complex. Telomerase activity is modified by enzymatic activities important in DNA-damage signaling such as p53. While it is evident that telomere shortening is critical to cell immortality, telomerase is likely to promote cell survival by mechanisms other than simply elongating telomeres, including genome stability (31).

### **MMR and Hereditary Nonpolyposis Colon Cancer**

The MMR system repairs single-base substitutions secondary to errors occurring during DNA replication. These errors, which occur at a higher frequency at mononucleotide or dinucleotide repeats due to DNA polymerase slippage, are thought to occur 1 per  $10^{10}$  events (Fig. 1). The errors are the outcome of the opposite effects of misincorporation of processive polymerase holoenzymes (1 per  $10^5$  errors) vs the combined efforts of the efficiency of editorial functions of polymerases (99.9%), mainly through their exonuclease function, and the efficiency of the MMR system (99%).

The MMR system in mammals is complex and is effected, in humans, by six MutS *Escherichia coli* homologs and three MutL homologs (33). The MMR system scans DNA in search of mismatches as well as of insertion/deletion loops ranging from 1 to 10 or more bases (34). MSH2 forms heterodimers with MSH3 and MSH6 that are able to recognize these errors. hMutS (hMSH2–hMSH3) and hMutL (hMSH2–hMSH6)

complexes have distinct specificity in error recognition (35). Once identified, a second complex formed by hPMS2 and hMLH1 completes DNA excision after strand discrimination. Recognition of the parental or correct strand is critical. While strand methylation plays a critical role in recognition in *E. coli*, the molecular basis of this process in mammals remains unknown (36). Finally polymerases, endonucleases, and other proteins contribute to complete the repair process. Again, the components of this repair pathway show complex interactions with other DNA repair proteins participating in the NER system (37) and the repair of DSB (34), suggesting additional roles in genome maintenance. These interactions may account for the observed resistance to some chemotherapeutic agents detected in cells with the MMR-deficient cells (38).

Inherited defects on the MMR machinery are responsible for a significant proportion of families diagnosed with hereditary nonpolyposis colorectal cancer (HNPCC) (39) (Table 1). This observation has been critical in strengthening the role of genetic instability in cancer development. A subset (10–20%) of colorectal (40) and a similar proportion of other types of human tumors (endometrial and gastric cancers) exhibit ubiquitous microsatellite instability (MSI). This discrete type of genomic instability is characterized by small deletions or insertions within short tandem repeats in tumor DNA compared with the matching normal tissue (41). MSI is the molecular symptom of a defective MMR machinery (42) and characterizes a distinctive tumor progression pathway known as the microsatellite mutator phenotype (43). In cells with one normal and one mutant allele of a DNA MMR gene, DNA repair is minimally impaired, if at all. When inactivation of the remaining allele occurs, however, many mutations in mononucleotide, dinucleotide, and trinucleotide repeat tracts (i.e., microsatellite sequence tracts) accumulate throughout the genome.

Germline mutations are mainly truncating point mutations, although gross deletions in *MSH2* and *MLH1* genes account for approx 40% of HNPCC as defined by Amsterdam criteria. Mutations inactivating other MMR genes (*PMS1*, *PMS2*, and *MSH6/GTBP*) have been seen in a small fraction of patients with HNPCC (44). Altogether, germline mutations in the known MMR genes have only been detected in 1–2% of patients with colorectal cancer, although approx 10–15% of all colon cancers display the MSI phenotype. In sporadic cases, the phenotype is due to inactivation of the *MLH1* gene that may occur by epigenetic changes, such as DNA methylation of *MLH1* transcriptional regulatory sequences (45,46).

While initially these alterations do not *per se* confer an advantage when these mutations occur within oncogenes or TSGs, they result in a selective growth advantage. Thus, e.g., in these tumors transforming growth factor- $\beta$  (TGF- $\beta$ ) type II receptors are frequently inactivated. The gene encoding the type II receptor contains an A<sub>10</sub> sequence that frequently undergoes mutation to A<sub>9</sub> or A<sub>11</sub> because of “slippage” of DNA polymerase during replication. If unrepaired by the MMR system, these mutations cause a frame shift in the protein-coding sequence that abolishes production of the normal receptor protein (46). As noted earlier, such inactivating mutations make cells resistant to growth inhibition by TGF- $\beta$ , thereby contributing to their unregulated growth. A similar situation has been reported for other genes involved in both DNA maintenance and controlling cell growth or apoptosis (47).

### **Other Types of Repair**

In addition to these coordinated repair pathways involving several components, single repair proteins can revert specific injuries. Alkylating agents induce mutations

and promote carcinogenesis, cell death, chromosome damage, and cell-cycle arrest. Alkylating agents transfer unsubstituted alkyl groups such as methyl or ethyl to nucleophilic sites in macromolecules. One of the many alkylation lesions in DNA is O<sup>6</sup>-alkylguanine (48). O<sup>6</sup>-metguanine is one of the most studied of alkyl-damaged bases. These lesions are highly mutagenic because AT transition mutations arise at sites of O<sup>6</sup>-alkylguanine after two cycles of DNA replication. The activity in charge of repairing this system was initially characterized as methylguanine-methyltransferases although it should be named alkyltransferase, because it removes all types of alkyl modification with distinct specificity. After repair, the enzyme is inactivated and committed to ubiquitination.

Alkyltransferase is frequently inactivated, mainly by epigenetic mechanisms, in several tumor types including colorectal (49) and central nervous system neoplasms (50), thus contributing to acquisition to additional genetic alterations. When adduct-promoting chemotherapeutic treatments are administered, lack of alkyltransferase may confer sensitivity to treatment with the tumor cell unable to repair the drug-induced DNA damage (49). This system requires a competent MMR system to be able to protect the cell adequately and reinforces the idea that distinct DNA repair systems must interact to maintain genetic instability.

### Chromosomal Instability

Specific functional defects in DNA repair can be associated with characteristic patterns of genetic instability: MSI secondary to failure in MMR pathways or NER failure associated with some rare cancer-prone syndromes. The underlying causes of more dramatic gross chromosomal alterations that predominate in human cancers are less clear. This disparity is quite striking because the karyotypic heterogeneity observed in most solid tumors should be considered among the most compelling evidence of the role of genetic instability in cancer development.

By analogy to MSI, Lengauer et al. (51) described chromosomal instability in colorectal cancer cell lines. Two types of chromosomal aberrations may be detected in human cancer cells: numerical and structural. Numerical changes are a common feature of human solid tumors and may arise as aneuploidies or polyploidies secondary to distinct mechanisms. Multiple karyotypic structural abnormalities are also typical of human neoplasms (52–54). In hematopoietic malignancies, structural alterations (translocations and inversions) have two principal consequences: gene activation or gene fusion (55). In most solid tumors, however, primary structural alterations have not been identified. It has been postulated that submicroscopic rearrangements may be early events (56), followed by unbalanced chromosomal abnormalities most likely associated with selective pressures during tumor progression.

The genetics of chromosomal instability remains a mystery. Although >100 genes can cause chromosomal instability in yeast, it is unclear whether one or a few master genes are responsible for most of the observed chromosomal instability in human cancer (57). It has been suggested that the *APC*, initially considered as a gatekeeper in colorectal tumorigenesis (3), should be viewed as a master in chromosome instability initiation in colorectal cancer (6,58). In mouse models, *APC* gene alterations result in early polyploid endoreduplication, a well-characterized process, but not in aneuploidy (increased losses or gains of one or a few individual chromosomes). After endoreduplication, the trend is toward chromosome loss (59). In colorectal cancer, this tendency could be attributed to second mutations in other genes.

A second class of chromosomal instability candidates comprises those genes involved in detecting spindle–kinetochore interaction during mitosis. The mitotic spindle checkpoint (57) and sister chromatid separation pathways (60) are well conserved in mammalian cells, suggesting that defects in such pathways can lead to types of chromosome loss seen naturally occurring in some cancers. *MAD* and *BUB* genes have been identified as key components of the mitotic spindle checkpoint in yeast. While human counterparts of these genes have been identified (61,62), the promise of identifying genes responsible for chromosomal instability in humans has not been completely fulfilled because only a few *BUB* mutations have been detected. Defects in the pathways operating downstream of the spindle checkpoint have been noted, mainly overexpression of hSecurin, a protein involved in the control of sister chromatid separation (63).

### Relevance of DNA Damage Sensing and Signaling

The first step in the response elicited by DNA damage includes sensing and recognizing DNA damage followed by activation of cell-cycle checkpoints. The importance of this step cannot be overemphasized. The defining feature of a cell-cycle checkpoint (64) is that it serves as a quality control system to couple sequential events within the cell cycle. Inadequate sensing and signaling of any DNA damage can be as harmful to genetic stability as any specific DNA repair defect (65). An ‘inappropriate’ repair can result in mutagenic repair of DNA damage associated with low apoptotic cell death (66). Consistent with this concept, most tumors show chromosomal instability and miss some checkpoints.

Three distinct DNA damage checkpoints act during cell-cycle progression: the G1/S-phase, to prevent from starting replication; intraS-phase, to prevent completing replication; and G2/M-phase, to prevent mitosis. We have previously discussed well-documented examples of the connection between DNA damage checkpoint genes (*ATM*, *BRCA1*, *BRCA2*, *NBS1*, *BLM*, and *WRN*) and tumorigenesis. Nevertheless, the list of these proteins is not complete until *p53* is added.

*p53* is a major genome guardian molecule (67) in response to DNA damage. After phosphorylation of specific residues by other signaling molecules (i.e., *ATM* gene product or Chk2 protein) (68), it mediates cell-cycle arrest (by inducing p21 expression) (69) or apoptosis after increasing bax expression (70), thereby preventing propagation of cells that have accumulated DNA damage. Under some circumstances, *p53* acts at the G1/S-phase checkpoint to regulate the cell’s decision to synthesize DNA, although *p53* also appears to have a critical function at the G2/M-phase checkpoint (71). *p53* is believed to be among the most frequently mutated genes in human cancer. Germline mutations in the *p53* gene have been seen in individuals affected by the Li–Fraumeni syndrome, a cancer-prone syndrome with a very high risk for the development of a number of tumors, including soft-tissue sarcomas, osteosarcomas, brain tumors, breast cancers, and leukemias (72). Li–Fraumeni syndrome provides genetic evidence for a common pathway of *p53* and *Chk2* genes. Germline mutations in the latter gene have been reported in families with Li–Fraumeni syndrome who are lacking *p53* mutations.

### Conclusion

The contribution of genetic instability to cancer development is well established and the contribution of caretaker TSGs to tumor development is evident. Specific functional defects in DNA repair can be associated with characteristic patterns of genetic instability that profoundly impact tumor biology. More information is needed about

the molecular basis of chromosomal instability, the putative underlying cause of all chromosomal alterations, either numerical or structural, present in tumor cells. It is noteworthy that the more is learned about DNA damage repair, the more complex the picture is and more interactions are depicted. Researchers need to gain insight into the molecular basis of sensing DNA damage and learn how cells discriminate between distinct types of damage and why they choose to use one repair system or another. Finally, because abnormal function of cell-cycle checkpoints may be as critical as specific DNA repair, defects in generating genetic instability should lead to efforts to improve knowledge about their function. A better comprehension of these mechanisms will certainly result in novel opportunities for better cancer prevention, management, and treatment.

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# III

## FUTURE DIRECTIONS

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# 16

## Immunotherapy for Human Cancer

### *Evidence and Obstacles*

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**Angelo A. Cardoso, W. Nicholas Haining, Marina Leite, and Sara Maia**

### **Introduction**

The human immune system can mount a specific response to cancer. Intense research is aimed at dissecting the intricacies of the interactions between the immune system and tumor cells. A driving force behind this research is the idea that cancer-directed immunity can be enhanced to improve the outcome for patients with the disease. The specificity of the immune response makes cancer immunotherapy extremely attractive because it offers the promise of reducing damage to bystander normal tissues and lessening the severe side effects associated with current cancer therapies. A variety of modalities can be incorporated into an immunotherapeutic approach, including vaccines, recombinant antibodies (Abs), cytokines, and cellular and gene therapies. Moreover, techniques are now available to measure antigen (Ag)-specific immunity with great sensitivity and allow the biology of tumor-specific T cells obtained from patients to be studied. These techniques provide the best evidence to date of the strengths and weaknesses of the human immune response to cancer. Formidable barriers remain, however, to the successful manipulation of tumor-specific immunity in cancer patients. This chapter focuses on recent developments in immunology that might signal the way to overcoming these obstacles.

### **Supporting Arguments**

For immunotherapy to work against cancer, a complicated series of cellular interactions must occur (1,2), including the following:

- Recruitment and engagement of the correct populations of immune cells with specificities for an array of immunogenic epitopes that cover the antigenic repertoire of a heterogeneous tumor
- Effective activation of the immune cells to maximize their effector potency
- Development of an efficient immunologic memory
- Blockade of inhibitory mechanisms that may limit the efficacy of the antitumor responses and facilitate tumor escape
- Elimination of putative immunoresistant phenotypes.

What is the evidence that any of these complex series of interactions occur in the tumor-bearing host and result in the generation of tumor-specific immunity?

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### ***Cancer Hosts Have Antitumor-Specific T Cells in Their Repertoire***

Studies in cancers of different histologies (melanoma; neuroblastoma; and breast, prostate, ovarian, colon, rectal, and bladder cancer) suggest that the presence of lymphocytes in the tumor microenvironment is associated with increased patient survival (3–13). Moreover, tumor-specific T cells can be generated or expanded from the peripheral blood, tumor-infiltrating lymphocytes (TILs), tumor-infiltrated lymph nodes (TILNs), bone marrow, tumor masses, and tumor exudates of patients with various tumor types (14–23). These findings show that most cancer patients have T cells that recognize antigens expressed by tumor cells, and that those T cells can proliferate and assume effector function. Moreover, it suggests that the host's immune system has at least launched an attempt to control the developing tumor, albeit unsuccessfully.

### ***Tumor Antigens Exist***

Tumor Ags are molecules that contain the epitope determinants recognized by T-cells that specifically target tumor cells. Cytotoxic CD8<sup>+</sup> T cells recognize short peptides (8- to 10-mers) presented in the binding groove of major histocompatibility complex (MHC) class I molecules (24–26). Helper CD4<sup>+</sup> T cells recognize larger peptides (14- to 28-mers) that bind to MHC class II molecules through their backbone residues (27,28). This specificity is dependent on the peptide sequence and the MHC allele, and it defines the population of T cells in an individual capable of responding to a given Ag. The demonstration that tumor Ags exist and that tumors may have antigenic signatures distinct from normal cells represented a major advance in tumor immunology and for development of immunotherapeutic strategies for human cancer (29,30).

Different methodologies have been developed to identify tumor Ags, including a variety of biochemical and genetic approaches, SEREX (serologic analysis of recombinant cDNA expression libraries), and reverse immunology (14,31,32). Algorithms for prediction of immunogenic peptides are publicly available (e.g., BIMAS HLA peptide-binding prediction system [33]; and SYFPEITHI [34]). To date, a significant number of tumor Ags have been identified and validated in preclinical studies. Extensive listings of tumor Ags, their peptide determinants, and their MHC restriction have been cataloged (35) and are available at Cancer Immunity ([www.cancerimmunity.org/peptidedatabase](http://www.cancerimmunity.org/peptidedatabase)), BIMAS ([bimas.cit.nih.gov/molbio/hla\\_bind](http://bimas.cit.nih.gov/molbio/hla_bind)), SYFPEITHI database ([www.uni-tuebingen.de/uni/kxi](http://www.uni-tuebingen.de/uni/kxi)), and Cancer Immunome Database ([www2.licr.org/CancerImmunomeDB/](http://www2.licr.org/CancerImmunomeDB/)).

Tumor Ags can be classified according to their source, pattern of expression, and tissue restriction. The SYFPEITHI database categorizes tumor Ags as melanoma- and prostate- differentiation Ags; cancer-testis Ags; nonmutated overexpressed Ags; mutated, unique Ags; and viral Ags. Most of the nonviral tumor Ags identified and validated by immunologic studies are molecules overexpressed in tumor cells but also present in normal cells, and molecules associated with the differentiation programs of particular cell lineages.

Optimal tumor Ags contain immunogenic epitopes that bind to MHC with high affinity and stability, and that are capable of recruiting an avid, broad, and functional T-cell repertoire that may mediate the elimination or containment of the tumor. Strategies have been devised not only to identify the most immunogenic tumor epitopes but also to modify subdominant or cryptic antigenic determinants to improve their immunogenicity (36). The rationale for this approach is that immunologic tolerance may exist to dominant tumor epitopes derived from molecules expressed by normal tissues. The cytotoxic T-lymphocyte (CTL) repertoire specific for several dominant tumor

epitopes from self-Ags is deleted or rendered ineffective (37–40). Because tolerance to self-Ags seems mainly to involve immunodominant determinants rather than cryptic determinants (41–43), subtle alterations in the peptide sequence of target Ag might recruit T-cells with sufficient T-cell receptor (TCR) avidity that they can kill tumor cells but not so much that they have undergone clonal deletion in the thymus (37,40,44). This strategy is generally referred to as the “heteroclitic peptide approach” and has allowed the generation of effective, potent responses to various tumors by recruiting CTL specific for modified, cryptic tumor epitopes (36,40,45,46).

### ***T Cell-Mediated Immune Responses Can Be Modulated***

Optimal T-cell-mediated responses to tumors will require the involvement of CD4<sup>+</sup> T-cells, which play a helper function, and CD8<sup>+</sup> T-cells, which are the cytotoxic effectors of the adaptive immunity (47,48). Most studies show that the recruitment and optimal expansion of tumor-specific CTLs mainly occurs through the presentation of immunogenic tumor epitopes through cross-priming, i.e., by professional Ag-presenting cells (APCs) that have engulfed the tumor Ag (49–52).

Elucidation of many of the principles regulating the effective priming, recruitment, and expansion of T-cells, paired with technologic advances in cell selection and manipulation, have facilitated the study of immune responses to tumors and the design of T cell-based immunotherapeutic strategies. Some of these principles and advances include:

- Differentiation, expansion, and maturation of APCs that can generate and amplify Ag-specific T-cell responses, particularly using dendritic cells (DCs) or, in a lesser degree, activated B cells (53–58)
- Optimization of strategies to deliver tumor Ag or epitopes into APCs or to augment the uptake machinery of APCs (59,60)
- Development of methods to improve the immunogenicity of APCs, namely by modulation of MHC, costimulatory, and adhesion molecules, and/or by the expression of relevant cytokines and chemokines. These changes may be accomplished using gene transduction (as viral vectors or naked DNA) or the use of recombinant molecules or agonists (cytokines, chemokines, fusion proteins, stimulatory Abs, or immune adjuvants) (61–65).

### **Obstacles**

Despite the presence of humoral and cellular tumor-specific immunity, most patients do not mount a sufficient immune response to rid themselves of cancer. To explain this conundrum, it has been proposed that tumor cells develop or use strategies to escape the putative surveillance activity of the immune system (Box 1). As most of these strategies have been extensively reported and reviewed, here we elaborate on the concept of tumor immunosurveillance and on some emerging mechanisms of immune escape in human cancer.

### ***Cancer Immunoediting: Tumor Immunosurveillance Revisited***

The concept that the immune system surveys the organism for emerging tumors and has the capacity to recognize and eliminate malignant cells is generally known as tumor immuosurveillance. The underlying principle is that cells of the immune system can recognize tumors as distinct from the normal tissues (non-self) or as danger to the organism.<sup>1</sup> Although it is well established that the immune system effectively surveys

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<sup>1</sup>It has been suggested that the immune system does not operate by discriminating self- and non-self but rather by responding to danger signals, which result from tissue distress (176).

**Box 1****Mechanisms of Tumor Escape From Immunity**

- Secretion of inhibitory cytokines that thwart immune function
- Paucity of chemokines that may attract immune effector cells to the tumor microenvironment
- Recruitment of accessory cells that directly or indirectly inhibit immunity
- Recruitment and activation of suppressive immune cells
- Induction or upregulation of surface molecules that inactivate immune effector cells
- Expression of tumor cells of molecules that induce apoptosis of immune cells
- Downregulation of molecules critical for antigen-presentation and costimulation of immune cells

for danger signals posed by invading pathogens, the existence of tumor immunosurveillance is the object of intense debate (2,66–70).

The strongest argument in favor of tumor immunosurveillance comes from studies showing an increased susceptibility to tumors in animals lacking critical elements of the immune system. Mice with severely compromised immune functions because of absence or functional inactivation of cells ( $\alpha\beta$  T-cells,  $\gamma\delta$  T-cells, natural killer [NK] cells, NKT cells) or cytokines (interleukin-12[IL-12], interferon  $\gamma$  [IFN- $\gamma$ ]) have a significantly higher susceptibility to develop carcinogen-induced tumors and spontaneous tumors (71–78). In humans, the strongest evidence is from analyses of cancer incidence in individuals with compromised immune systems as a result of either genetic or acquired immunodeficiencies. A high incidence of viral-induced tumors is observed in immunocompromised individuals (79–82), which supports the critical role of the immune system in the surveillance of infectious agents. More importantly, a significantly higher frequency of nonviral cancers was observed in immunosuppressed individuals (69,79,80,83).

An immune system that can detect early tumors and eliminate them implies that emergent malignant cells are able to evade or overcome the selective pressure of immunity, to evolve into a “successful” cancer. This immune evasion—the selection of tumor variants that escape the immune system—is a strategy used by certain pathogens to attain selective advantage in infected hosts. This multistep phenomenon is called immunologic sculpting and suggests that immunoediting of tumors occurs by a sequence of events that involves elimination of the immune-sensitive tumor cells, followed by an equilibrium phase in which immune-resistant tumor cells are selected, and, finally, an escape phase in which the immune-selected tumor variants expand to later become a clinically detectable cancer (69).

An alternative view is that tumors are perceived by the immune system as normal tissue and, therefore, do not release danger signals that may trigger an immune response (2). In this model, no immune sculpting forced by antitumor immunologic pressure occurs, and the appearance of tumor cell variants is rather due to a natural selection of tumor subclones generated by genetic instability and dysregulation of DNA repair (2).

The idea that the immunoresistant variants emerge during tumor progression has important implications for the development of immunotherapeutic strategies for human cancer. First, if immune sculpting selects nonimmunogenic variants, conventional immunotherapeutic strategies are likely to be ineffective, and alternative approaches might be necessary to target more efficiently these immunologically silent tumors.

**Table 1**  
**Human NK Cell Receptors (NK Receptors)<sup>a</sup>**

Receptors	Ligands	Expression
Binding MHC I molecules—NKR (classic and nonclassic)		
KIR	HLA-A, -B, -C, -G	NK cells; CTLs
CD94/NKG2	HLA-E	NK cells; CTLs
ILT/LIR	HLA-A, -B, -G1 UL18 glycoprotein	NK cells; CTLs
NKG2D	MICA, MICB, ULBPs	NK cells; CTLs
Binding non-MHC I molecules		
NCR	Non-MHC molecules	NK cells

<sup>a</sup> Receptors that bind MHC class I molecules are known as NK receptors (NKR). KIR, killer Ig-like receptor (13 known members; belong to Ig superfamily); ILT/LIR, Ig-like transcripts/leukocyte Ig-like receptor (only CD85j/ILT2 function as NKR); NKG2 (7 known members; belong to C-type lectin superfamily); NCR, natural cytotoxicity receptors (4 known members), which have been identified as activating receptors that lyse MHC I-negative target cells.

Second, even if cellular immunotherapy is effectively targeting and eliminating tumor cells, it may drive the selection of nonimmunogenic, treatment-insensitive tumor variants. This fact may contribute to the generally limited efficacy of most immunotherapeutic approaches under clinical evaluation and needs to be considered in the design of novel strategies for cancer immunotherapy.

**Tumor Cell Evasion: Physiology and Deception**

Different mechanisms have been implicated in the resistance of tumors to immune attack. Most of these mechanisms or strategies are not tumor specific but, rather, represent the use by tumor cells of biologic tools also used by normal cells or in effect in the microenvironments where tumor cells develop or invade. In other words, tumors use physiology to elude immunity.

**Modulation of NK Receptors**

NK cell receptors are molecules that regulate the cytotoxic function of NK cells and CTL (for comprehensive reviews, see refs. 84–87). In humans, these NK cell receptors can be divided into two groups: receptors whose cognate ligands are classic or nonclassic MHC class I molecules, and receptors whose cognate ligands are non-MHC class I molecules. Table 1 summarizes some of the properties of these receptors. For the sake of simplicity, we hereafter refer to all NK cell receptors as NK receptors, whereas the subgroup of receptors whose cognate ligands are MHC class I molecules is referred to as NKR.

The NKR families (such as killer immunoglobulin [Ig]-like receptor [KIR] and C-type lectin receptors) include both receptors that inhibit and receptors that activate the lytic function of immune cells. The inhibitory receptors contain immunoreceptor Tyr-based inhibitory motifs (ITIMs) in their cytoplasmic domain, whereas the activating receptors do not. The function of the activating receptors depends on their association with an adapter molecule such as DAP12, which has immunoreceptor Tyr-based activation motifs (ITAMs) (88,89). On ligand binding to activating NK receptors, the Tyr residues in the ITAM are phosphorylated and trigger NK cell activation (88).

Conversely, ligand binding to inhibitory NK receptors leads to Tyr phosphorylation of ITAM and recruitment of tyrosine phosphatases that block the signals from activating receptors. Therefore, the activity of cytotoxic cells, which may simultaneously express a variety of inhibitory and activating NK receptors, is regulated by a balance between negative and positive signals transmitted upon ligation of their cognate ligands on target cells. This balance is weighted in favor of inhibitory signals that are dominant over the activating ones.

ILT2, similarly to inhibitory KIR, mediates inhibition of NK cell-mediated cytotoxicity and increases the threshold for CD8<sup>+</sup> T-cell activation in ILT2-positive T cells (90). The function of CD94/NKG2 receptors is defined by which one of the family of NKG2 molecules dimerizes with CD94. If the dimer is with NKG2A or NKG2B, inhibitory receptors are formed. Conversely, if CD94 dimerizes with NKG2C, E, F, or H, activating receptors are formed. Although functioning as an activating receptor, NKG2D is distinct from other NKG2 family members in that it forms a homodimeric structure and, unlike other activating NKRs, is associated with a different adapter molecule—DAP10 (91). The ligands for human NKG2D include the polymorphic nonclassic MHC I molecules, MICA and MICB, which are expressed in response to stress conditions (92), and the UL16-binding proteins (93). Engagement of NKG2D directly activates NK cells, whereas on T-cells it seems to provide a costimulatory signal (94–96).

Different mechanisms have been proposed that implicate NK receptors in tumor cell escape from immunity, including the following:

- *Secretion of soluble ligands for NKR.* Tumors of epithelial origin produce a soluble form of MICA, a ligand for the activating receptor NKG2D expressed by NK cells and CTLs (97,98). In melanoma and breast, colon, lung, and ovarian cancer, increased amounts of soluble MICA have been observed in patients' serum (98). This represents a novel mechanism for tumor escape since the soluble MICA saturates the NKG2D present on cytotoxic cells (97) and reduces NKG2D expression through endocytosis and degradation after MICA binding (98). This process is further illustrated by the observation that TILs have a reduced expression of NKG2D (98).
- *Imbalanced expression of inhibitory NKR.* CTLs present in lymphocyte infiltrates from patients with renal cell carcinoma expressing the KIR p58 show low antitumor lytic activity (99,100). The inhibitory effect of the p58 receptor requires its engagement by its cognate ligand expressed by tumor cells (99), hence suggesting a novel mechanism of tumor escape in which tumor cells regulate KIR inhibitory function on CTLs thus controlling the tumor host's cytotoxic responses. Neutralizing experiments using anti-p58 monoclonal antibodies showed an increased CTL activity directed to autologous tumor cells (100). In patients with melanoma, tumor-specific CTLs express the inhibitory receptor CD94/NKG2A and are ineffective in lysing melanoma cells, suggesting that in vivo modulation of NKR may decrease tumor-specific CTL activity (101).
- *Altered expression or function of NKR.* CD8<sup>+</sup> T cells from patients with multiple myeloma express increased amounts of CD94 but, in most cases, lack expression of their dimerization partners NKG2A or NKG2C (102). The presence of these abnormal forms of CD94 receptors may affect the ability of T cells to fine-tune their activity, and the absence of activating NKR (as CD94/NKG2C dimers) may impair the activation of tumor-specific T cells with low-affinity TCR (102).
- *Altered expression or function of NCR.* The defective antileukemia NK cell activity observed in patients with acute myeloid leukemia may result from the defective expression of natural cytotoxicity receptor (NCR) (103). It is also suggested that tumor cells express insufficient levels of NCR ligands, which may constitute a mechanism of tumor



escape, possibly explaining why, in some patients, although NK cells express high levels of NCR (NCR<sup>bright</sup>), a defective antileukemia cytotoxic activity is also detected (103).

Additional studies showed the expression of inhibitory NKR by TILs present in different tumors, though no formal demonstration of a potential tumor escape activity has been shown (101,104–108). Although cytokines IL-12, IL-15, and transforming growth factor- $\beta$  (TGF- $\beta$ ) can induce the expression of CD94/NKG2A in T cells in vitro (109,110), the mechanisms regulating the expression in vivo of NK receptors in cytotoxic immune cells are still largely unknown. In particular, it is not known whether NK receptor expression can be modulated by tumor cells, accessory cells, or cytokines present in the tumor microenvironment, or whether T cells expressing defined NK receptors are selectively recruited to the tumor sites. The modulation of NK receptors could offer significant therapeutic advantages as upregulation of activating NKR and/or downregulation/blockade of inhibitory NKR could improve the efficacy of antitumor cytotoxic cells and thus enhance the overall potency of immunotherapeutic strategies.

### **Regulatory T Cells**

Regulatory T cells (T<sub>Reg</sub>) are a subset of T lymphocytes that exert a suppressive activity on T cells, both in vitro and in vivo. T<sub>Reg</sub> cells have a major role in modulating T cell-mediated responses to self- and foreign Ag and in the unresponsiveness to nondanger signals (111–114). Increasing evidence shows the correlation between quantitative and functional dysregulation of T<sub>Reg</sub> cells and the development of autoimmunity and inflammatory diseases, as well as an altered peripheral T-cell homeostasis (114,115). T<sub>Reg</sub> cells have important roles in allergic reactions, transplantation tolerance, and tumor immunity (112,115,116).

Although some heterogeneity may exist (117), human natural T<sub>Reg</sub> cells are phenotypically characterized as CD4<sup>+</sup> CD25<sup>high</sup> cells exhibiting features of both anergic and activated T cells including the constitutive expression of CTLA-4/CD152, CD45RO<sup>high</sup>, CD45RA<sup>low</sup>, CD122/IL2-R $\beta$ <sup>low</sup>, CD69<sup>high</sup>, and production of IL-10, TGF- $\beta$ 1, and TGF- $\beta$ 2 (118,119). These cells appear to be long-lived, have a reduced proliferative potential, and are resistant to apoptosis. Most natural T<sub>Reg</sub> cells do not show simultaneous expression of CD25/IL2-R $\alpha$  and CD122/IL2-R $\beta$ , which may explain their poor responsiveness to IL-2. T<sub>Reg</sub> cells suppress activation of CD8<sup>+</sup> T cells in a contact-dependent manner, resulting in the inhibition of IL-2 production and upregulation of CD25 (120). To exert their immunosuppressive activity, T<sub>Reg</sub> cells require activation, after which they undergo phenotypic changes that include the increased expression of CTLA-4 and CD122/IL2-R $\beta$  and the increased production of IL-10.

Increasing evidence suggests that T<sub>Reg</sub> cells may play a critical role in cancer development and tumor immunity. The underlying idea is that immunoregulatory T cells, by serving their physiologic function of protecting the host from unwanted immunity to self-Ag, may lead to failure of immune surveillance of tumor cells.

In colorectal carcinoma, it has been observed that a tumor-reactive T<sub>Reg</sub> clone suppresses the proliferation of both autologous antitumor T cells and mitogen-activated allogeneic T-cells (121). More important, these T<sub>Reg</sub> cells also inhibit the ex vivo generation or expansion of autologous tumor-specific CTLs. This immunosuppressive effect is mediated by TGF- $\beta$  and, atypically, does not seem to require cell–cell contact (121). In pancreatic and breast cancer, Liyanage et al. showed an increased frequency of T<sub>Reg</sub> cells in the peripheral blood and their presence in tumor sites and regional lymph nodes. These T<sub>Reg</sub> cells secrete IL-10 and TGF- $\beta$  and are potent inhibitors of cell prolif-

eration and IFN- $\gamma$  secretion by primed CD8<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>-</sup> T cells. In lung cancer, tumor sites contain large numbers of T<sub>Reg</sub> cells (123,124) that selectively inhibit the proliferation of autologous T cells but not allogeneic T-cells (124). In our own studies, we observed the presence of immunoregulatory CD4<sup>+</sup>CD25<sup>+</sup> IL-10-producing T-cells in the bone marrow of patients with leukemia and showed that in vitro culture with leukemia cells increased the relative frequency of the T<sub>Reg</sub> cells (125). These studies suggest that T<sub>Reg</sub> cells present in the leukemia microenvironment may mediate the “linked suppression” of antitumor T cells by juxtacrine production of inhibitory cytokines (submitted for publication).

In murine tumor models, elimination of T<sub>Reg</sub> cells results in potent immune responses to syngeneic tumors and, frequently, in tumor rejection. Removal of immunoregulatory CD4<sup>+</sup> CD25<sup>+</sup> T cells overcomes the immunologic unresponsiveness to tumors in vivo and in vitro (126). In a murine model of melanoma using B16 cells, depletion of T<sub>Reg</sub> cells prevented the growth of malignant cells in most animals and resulted in long-lasting protective tumor immunity (127). Another study concluded that the combination of T<sub>Reg</sub> depletion and CTLA-4 blockade resulted in maximal CTL-mediated rejection of melanoma cells (128).

These studies suggest that T<sub>Reg</sub> cells may allow tumor cells to escape the tumor host's immune surveillance by suppressing the induction or recruitment of antitumor CTLs. These findings disclose the principle that neutralization or elimination of immunoregulatory T cells may constitute valid therapeutic approaches for the development of more effective antitumor immunotherapy. A note of caution, however—it is questionable whether the functional blockade or elimination of T<sub>Reg</sub> cells will be sufficient to promote regression of established tumors.

### **Tumor-Associated Macrophages**

Increasing evidence indicates that macrophages that infiltrate the tumor microenvironment—tumor-associated macrophages (TAMs)—have a critical role in tumor proliferation and progression (129–131). In some cancers, macrophages constitute a significant part of the tumor microenvironment, to where they are recruited by chemokines (such as MCP-1/CCL2, RANTES/CCL5, GRO $\alpha$ /CXCL1) and cytokines (vascular endothelial growth factor, macrophage colony-stimulating factor) produced by tumor cells or other tumor-accessory cells, such as endothelial and stromal cells. An important component of the biologic effect of TAMs in tumor development is their impact on tumor cell survival and proliferation and is exerted through the production of tumor-stimulating cytokines (132), modulation of the extracellular matrix (129,133,134), and promotion of neovascularization (135–139).

Although it has been suggested that macrophages present in tumor sites may mediate tumor cytolysis (140), TAMs are poor APCs and are unlikely to play any significant role in the generation of antitumor CTL responses. Rather, evidence supports the concept that TAMs facilitate tumor progression by impairing tumor immunity. TAMs suppress the activation of T cells through the production of cytokines (such as IL-10 and TGF- $\beta$ 1) and prostaglandins (141–143). The elucidation of the role(s) that TAMs play in supporting tumor progression may provide therapeutic targets for novel treatment approaches.

### **Dissecting the Spontaneous T Cell Response to Human Cancer**

The existence of tumor-specific T cells in patients with cancer has been recognized and documented for well over a decade. Little is known, however, about the initiation or control of this type of immune response. Moreover, with a few notable exceptions,

the antitumor immune response has proven difficult to manipulate by conventional immunotherapies, such as vaccination. Nevertheless, the specificity and potency of T-cell-mediated cytotoxicity makes the induction of a robust immune response against cancer a highly desirable therapeutic goal.

The pursuit of clinically meaningful antitumor immunity has been accelerating markedly in recent years, fueled largely by observations made using animal models of cancer. Until recently, these observations were difficult to translate to the clinical realm for several reasons. First, T-cells specific for Ags expressed by tumor cells are very rare in the bloodstream, making their study technically challenging. Second, relatively few Ags that elicit T-cell responses in humans have been identified. The last decade, however, has seen a dramatic increase in the number of tumor Ags that generated measurable humoral and/or cellular immunity in cancer patients (35). More important, an enlarging armamentarium of techniques has been developed that allows weak antigen-specific T-cell responses to be detected and characterized *ex vivo*.

### **Techniques to Detect Ag-Specific T Cells**

Thierry Boon and colleagues pioneered the classic approach to identifying tumor-specific T cells. (144). T-cells isolated from TILNs or tumor deposits were repetitively expanded in the presence of autologous tumor cells and fed with exogenous cytokines. This technique allowed the selective enrichment and subsequent cloning of T-cells specific for autologous tumor targets. Boon et al. (144) used these clones, together with molecular biologic techniques, to identify the tumor-associated Ag recognized by the clones. While T-cell clones retain the specificity of the parental T-cell, the phenotype, function, and biology are markedly altered by the technique of cloning, and very little information was preserved about the functional state of the parental T-cells from which the clones were generated. The corollary was, until recently, that without progressive rounds of *ex vivo* expansion, the frequency of tumor-specific T-cells in patients was too low to be detectable. The development of new methods such as the ELISPOT assay, HLA-tetramers, and refinements in intracellular cytokine staining, however, have allowed the detection and characterization of low-frequency T cells (Fig. 1).

### **ELISPOT Assay**

The ELISPOT assay is a development of the enzyme-linked immunosorbent assay technique for quantifying cytokine release in which individual T-cells stimulated by an Ag are detected by the secretion of cytokine (145,146). Bulk populations of peripheral blood cells are cultured for short periods (typically overnight) in Ab-coated wells, with a source of Ag of interest. When T cells specific for the Ag are activated, they secrete cytokines such as IFN- $\gamma$ , which is trapped by the Ab coating the well. The cells are then washed off and the footprints of individual, activated T-cells are visualized with a secondary Ab linked to a colorimetric reagent. These spots can be digitally imaged and quantified and are interpreted as the number of T-cells specific for the Ag (147). This method is well suited to analyze large numbers of samples and can be used successfully with cryopreserved samples. The ELISPOT assay is frequently used to quantify the frequency of T cells specific for peptides corresponding to Ags used in tumor vaccines (148–152). The limitation of the ELISPOT assay is that it relies on the function of Ag-specific T-cells to allow their detection. T cells that have specificity for an Ag but are not activated by it will not be detected. In addition, the assay is not suited to the characterization of Ag-specific T cells beyond their ability to secrete cytokines.

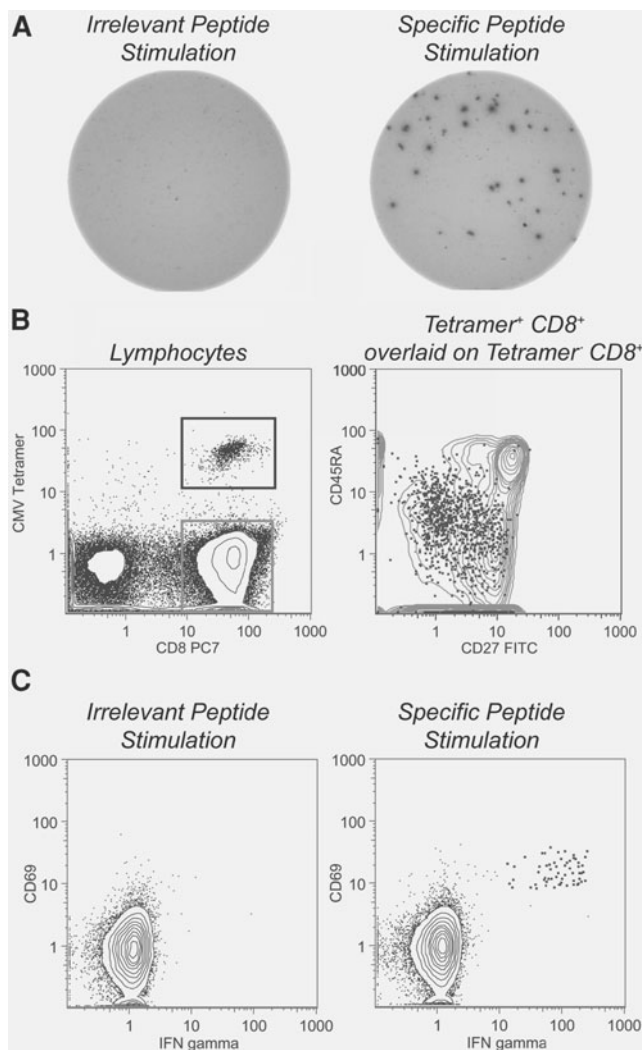


Fig. 1. Assays for characterizing antigen-specific immunity. Peripheral blood mononuclear cells (PBMCs) from an HLA-A\*0201<sup>+</sup> cytomegalovirus (CMV) seropositive healthy donor were used for three assays. **(A)** ELISPOT assay. PBMCs were cultured overnight with an irrelevant peptide (**left**) or an HLA-A\*0201-restricted epitope from the CMV pp65 protein (**right**). The dark spots visualized represent individual, activated Ag-specific T cells. **(B)** HLA-tetramer assay. PBMCs were stained with Abs against CD8, CD45RA, and CD27 together with an HLA-tetramer specific for T cells specific for the same peptide epitope as in **(A)**. (**Left**) A population of tetramer<sup>+</sup> CD8<sup>+</sup> cells (black box) can be easily distinguished from tetramer<sup>-</sup> CD8<sup>+</sup> cells (gray box). (**Right**) The pattern of staining of CD45RA and CD27 is different for tetramer<sup>-</sup> cells (gray contour plot) compared with tetramer<sup>+</sup> cells (black dot plot overlay). CMV-specific T cells (tetramer<sup>+</sup>) are predominantly CD45RA<sup>dim</sup> and have heterogeneous staining for CD27. CD45RA<sup>+</sup> CD27<sup>+</sup> (naive phenotype) cells are absent from the tetramer<sup>+</sup> population. **(C)** Intracellular cytokine staining. PBMCs were cultured for 6 h with an irrelevant peptide (**left**) or with the pp65 peptide (**right**) in the presence of Brefeldin-A and CD28 Ab. After incubation, cells were fixed, permeabilized, and stained with Abs to CD8, CD69, and IFN- $\gamma$ . The contour plots shown are gated on CD8<sup>+</sup> cells. A population of activated, CD69<sup>+</sup> IFN- $\gamma$ <sup>+</sup> cells is seen in the right panel (bold dots).

### **HLA-Tetramers**

Most T cells recognize peptides (derived from pathogenic organisms, self-proteins, or mutated proteins) displayed in the context of MHC I on the surface of target cells or APCs. This recognition is specific for both the MHC allele and peptide involved. Synthetic complexes of the relevant MHC allele complexed to a peptide Ag bind to the TCR of cells specific for that peptide–MHC complex. The binding of monomeric peptide–MHC complexes is weak, but if the complexes are multimerized (typically in tetramers), the binding can be enhanced by the cooperative binding of TCR (153). Linking tetrameric complexes to fluorochromes allows the flow cytometric detection of extremely rare populations of Ag-specific T cells without successive rounds of ex vivo expansion. The concomitant use of multiparameter flow cytometry allows the expression level of several surface or intracellular proteins to be measured simultaneously. This technology has led to an increasingly sophisticated analysis of the life cycle of T-cells specific for tumor Ags (154).

### **Intracellular Cytokine Staining**

Intracellular cytokine staining is another technique that allows the detection of T cells specific for an Ag on the basis of their function. Although the use of flow cytometry to measure expression levels of intracellular proteins is not a new technique, it has become more useful for quantifying Ag-specific T cells by the addition of a short in vitro stimulation with a peptide Ag of interest before staining. The in vitro stimulation is done in the presence of an inhibitor of protein secretion to increase the amount of cytokine in activated cells. This method sensitively identifies rare populations of Ag-specific cells on the basis of cytokine synthesis and can also be coupled with multiparameter flow cytometry to probe the biology of the activated cells. T cells stimulated with a peptide Ag and stained for intracellular cytokines can be colabeled with relevant tetramers to provide the greatest amount of information regarding the functional status of Ag-specific cells (155).

The increasing availability of these techniques has allowed considerable insight into the biology of tumor-specific T cells in patients with cancer.

### **Frequency of Tumor-Specific T Cells**

The best-studied T-cell responses are against melanoma Ags (156). It is unclear whether melanoma is inherently more immunogenic than other tumors or whether technical factors have led to the identification of more Ags in this type of cancer. Most of the Ags currently under study were discovered through the use of tumor-specific T-cell clones. While this confers biologic validity to those antigenic targets, it does not guarantee that they are the sole or immunodominant Ags, let alone those most relevant for a clinically meaningful immune response against the disease. Moreover, the restricted expression of melanoma Ag to that disease alone limits the broader conclusions that can be made regarding T cell responses to cancer. Technical difficulties exist with quantifying tumor-specific T cells. Their frequency is so low as to be at the limit of detection for even the more sensitive assays such as tetramer or ELISPOT. This means that the difference between a detectable immune response and a nondetectable one might lie in slight variations in experimental techniques among studies. Few groups publish a rigorous quantification of background levels of Ag-specific T-cells in control subjects where the assay would be expected to yield a null result, which limits cross-comparison of studies performed by different investigators.

**Table 2**  
**Frequency of Tumor-Specific T Cells in Patients with Cancer<sup>a</sup>**

Disease	Ag	Detectable with in vitro stimulation (proportion of patients)	Detected without in vitro stimulation (max. reported) (% of CD8 <sup>+</sup> )		References
			Blood	TILNs	
Melanoma	NY-ESO1	5/10	0.04	—	(177)
Melanoma	MAGE	11/22	ND	—	(178)
Melanoma	Melan-A	11/12	0.23	12	(165,174, 179–183)
Melanoma	Tyrosinase	6/10	2.2	0.8	(165,184)
CML	Proteinase 3	—	1.5	—	(160)
Solid tumors, myeloma	Telomerase	6/6	ND	—	(185)

<sup>a</sup> ND, nondetectable; CML, chronic myelogenous leukemia.

Ex vivo expansion with a source of Ag and exogenous cytokines is often used to increase the frequency of the T-cells above the threshold of detection, which limits cross-comparisons, and is likely to perturb the biology of T cells being studied.

Despite these drawbacks, several studies have attempted to measure the size of spontaneous immune responses to melanoma as well as other types of cancer (Table 2). For the most part, these studies have established that the frequency of T cells specific for cancer-related Ags is very low. The best-characterized Ag is the Melan-A/MART-1 epitope, largely because the frequencies of T cells specific for this Ag are higher than for most other Ags tested. The size of the T-cell response, however, is considerably smaller than that elicited by viral Ag. For instance, after infections with Epstein–Barr virus (EBV), as much as half of the T-cell compartment can be comprised of T cells specific for EBV Ag (157,158).

The frequencies of T cells specific for melanoma Ag vary considerably by site. TILN have significantly higher frequencies of tumor-specific T cells than peripheral blood. This compartmentalization is not surprising given the evidence from studies of viral infections that show marked segregation of T-cells in different organ beds (159). The higher frequency of tumor-specific T cells in lymph nodes infiltrated by cancer cells is intuitive given that the lymph node provides a fertile environment for the cross-presentation of Ags from dying tumor cells to T cells. The fact that the peripheral blood has a lower fraction of tumor-specific T cells has potentially serious consequences for therapeutic studies that typically use peripheral blood as a source of T cells to use as surrogate markers of efficacy. Difficulties in measuring tumor-specific T cells in the blood, as opposed to tumor bed, or TILNs may be more of an issue in solid tumors than in hematologic malignancies such as chronic myelogenous leukemia (CML) (160).

The magnitude of the T-cell response is also affected by the immunologic status of the patients studied. For instance, the proportion of patients with CML who have T cells specific for the CML antigen proteinase 3 is much lower among those treated with chemotherapy compared with those treated with IFN- $\gamma$  (160). The reasons for the difference are likely to be related to host factors such as disease burden and the immunosuppression caused by chemotherapy. Many other studies of tumor immunity have used samples from patients with a range of disease burden and prior treatment, which makes generalizations about the immunogenicity of tumors difficult.

The relatively high frequencies of T cells for the melanoma Ag Melan-A may be explained in part by the surprising observation that many healthy people have detectable numbers of Melan-A-specific T cells in their peripheral blood. The frequencies seen are similar to those in patients with melanoma, but, unlike those in cancer patients, they have a phenotype consistent with naive T-cell status. It is unclear why normal individuals should have T cells specific for a self-Ag, but their naive phenotype suggests that they are not the result of expansion in response to Ags; rather, they may represent a high natural frequency of a TCR with specificity for that Ag (161,162). This observation is supported by the finding of relatively high frequencies of Melan-A-specific T cells in very immature thymic T cells, suggesting that there is an absence of clonal deletion of cells specific for this Ag in the thymus (163). Alternatively, some environmental Ags may have sufficient similarity to the peptide sequence of Melan-A to allow them to be cross-recognized by Melan-A-specific T cells (164). Persistent low-level exposure to these Ags might sustain Melan-A-specific T cells at frequencies higher than for other Ags that have no similarity to environmental Ags.

The data regarding TCR diversity among cells specific for tumor Ags are contradictory. Some studies show a narrow, almost clonal TCR distribution (165), whereas others have found a relatively large repertoire of TCR used (166).

### ***Phenotype and Function of Tumor-Specific T Cells***

Tumor-Ag-specific T-cells can have several different maturational states. Naive T cells express CD45RA together with other surface markers such as CCR7 and CD62L that serve to target them to secondary lymphoid areas where they are most likely to encounter Ags presented to them by DCs (167–170). After encountering Ags in the correct immunologic milieu, Ag-specific T cells proliferate and develop effector functions. A portion of Ag-primed (or experienced cells) circulate in the peripheral blood as memory cells that are capable of a vigorous response on secondary challenge with Ag. Studies of memory T cells for viral Ag show that most cells lose expression of naive markers CCR7 and CD62L, and gain expression of CD45RO. A proportion of memory T cells, however, retain CD45RA expression and can have variable expression of CD27 and CD28 (171,172). Most studies addressing memory T-cell phenotype have addressed CD8<sup>+</sup> T cells exclusively. The phenotype of memory CD4<sup>+</sup> T cells is not as well characterized.

Most studies of tumor Ag-specific T cells have found that at least a proportion of them has a memory phenotype (165,173). Some patients with melanoma, however, have circulating Melan-A-specific T cells with a naive phenotype (CD45RA<sup>+</sup>/CCR7<sup>+</sup>) (161,173,174). This naive phenotype is shared by Melan-A-specific T-cells detected in normal donors (161,173,174). It is tempting to speculate that some individuals who did have naturally occurring Melan-A-specific T cells go on to develop melanoma, then exposing those T cells to a large source of antigenic challenge. Exposure to melanoma Ags in the right immunologic context might allow the preexisting naive T cells to develop an Ag-experienced memory phenotype. However, one study found no correlation between the frequency of Melan-A-specific T cells and a memory phenotype, suggesting that even if the T cells do encounter Ag, they may not proliferate significantly in patients with cancer (161).

The function of T cells specific for Melan-A is related to their phenotype. Cell lines and clones cultured from sorted tetramer-positive cells from patients with melanoma and normal donors can proliferate strongly and have brisk cytotoxic activity against

tumor cell targets (173,175). In Melan-A-specific T-cells that have not been expanded in culture, the ability to secrete cytokines in response to an antigenic challenge is markedly diminished in cells that have the naive phenotype of CD45RO<sup>-</sup>/CD45RA<sup>+</sup>/CCR7<sup>+</sup>. Healthy control patients, whose Melan-A-specific cells were predominantly of a naive phenotype, had no response in an ELISPOT assay using a Melan-A peptide challenge (174). This result is in keeping with the theory that these Ag-specific T cells in healthy donors and in some patients with melanoma are naive cells that have not been exposed to Ag previously.

## Conclusion

Recent progress in our understanding of the human immune response to cancer has been driven by three main factors: increased understanding of the regulation of immune responses to specific Ags, broader knowledge of immunogenic cancer Ags, and enhanced techniques to measure tumor-specific immune responses in patients with cancer. Important questions remain largely unanswered, and, to date, few patients have been helped by immunotherapies. Future research will need to address critical issues such as the impact of the tumor microenvironments on the ability of the immune system to recognize cancer cells and how the microenvironment contributes to immunoselection. Elucidation of phenotypic and antigenic changes in early stages of the malignant transformation will also shed light on the immunogenicity of nascent tumors, as well as on their ability to trigger tissue damage and inflammatory reactions. It will also be important to know whether putative immune reactions to emerging tumors engage mainly innate immunity, which exerts a nonspecific selective pressure, or also involve adaptive immunity, which may select against more immunogenic tumor cells and spare nonimmunogenic tumor cells. The factors that allow some immune responses to generate long-lasting potent effector T-cell memory and others to fade away need to be identified. Finally, the requirements to overcome the mechanisms that presumably exist to prevent immune responses to self-Ag must be characterized. Only then will potent immunity to tumor Ags—which are mostly self-Ags—be possible. Dissection of these questions will significantly contribute to the knowledge of tumor immunosurveillance and tumor immunity to human cancers, and will hopefully result in the design of more effective treatment strategies.

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## Treatment with Monoclonal Antibodies

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### Monoclonal Antibody Therapy: General Overview

#### ***Antibody Structure***

All intact therapeutic antibodies (Abs) now on the market are of the IgG class. IgG molecules are often depicted as Y-shaped structures. While not a true representation of its tertiary structure, the Y shape accurately represents the key features of an IgG molecule. Essentially, an Ab contains three components—two identical Fabs (for fragment–antigen [Ag] binding, the arms of the Y) and an Fc (for fragment crystallizable, the stem of the Y). Each Fab contains an Ag-binding site. The Fc contains structural features that determine the downstream consequences of Ag binding, often called the effector function of the Ab. For example, the Fc portion determines whether an Ab binding a cell-surface receptor simply prevents signaling through that receptor or, alternatively, causes the cell's destruction through complement fixation or targeting immune effector cells.

Each IgG molecule contains two identical polypeptide heavy chains and two identical light chains. Each heavy chain has four domains of approx 100 amino acids each, and each light chain has two domains. Typically, each domain is constrained by a single internal disulfide bond. The heavy chain domains, from amino terminus to carboxy terminus, are designated as  $V_H$ ,  $C_{H1}$ ,  $C_{H2}$ , and  $C_{H3}$ , where V denotes variable and C denotes constant regions. Similarly, the light chain domains are designated as  $V_L$  and  $C_L$ . The intertwining of a  $V_H$  and a  $V_L$  domain forms each Ag-binding site. Each V domain contains three short stretches of peptide, known as complementarity-determining regions (CDR). These regions, on folding, contribute to the Ag-binding pocket. Interchain disulfide bonds between  $C_{H1}$  and  $C_L$  stabilize the heavy and light chain association. Multiple inter-heavy chain disulfide bonds are located at the hinge region, which resides between  $C_{H1}$  and  $C_{H2}$ . The hinge region determines the flexibility of the Fabs relative to the Fc and may, therefore, affect the extent to which an Ab can bind to two Ags simultaneously.

In addition to protein, Abs have a carbohydrate component. Each heavy chain of an IgG molecule contains an N-linked carbohydrate moiety in  $C_{H2}$ . The specific composition of the carbohydrate moiety can be critical to the Ab's effector function (1,2).

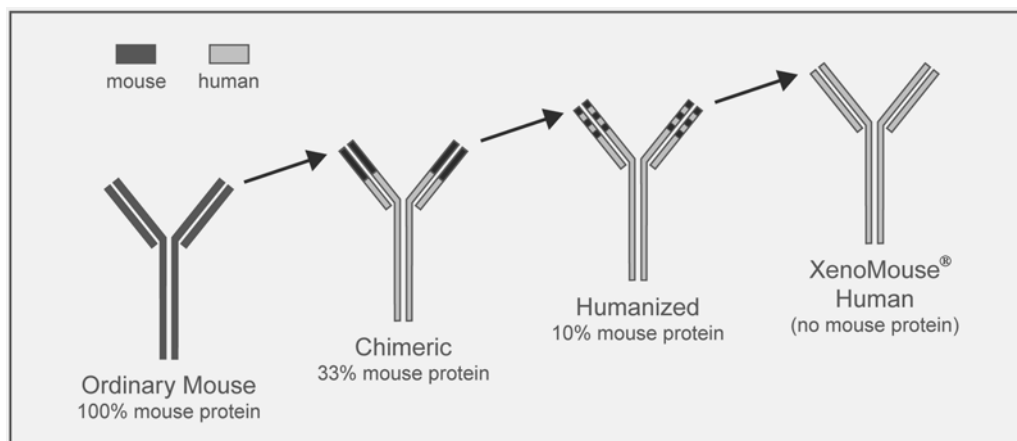


Fig. 1. Evolution from fully murine to fully human MAbs.

## Antibody Generation

### Technical Approaches

The single biggest driver for the approval of 14 Abs as therapeutics over the past decade has been the advance of technologies for Ab generation. While the development of hybridoma technology to produce monoclonal antibodies (MAbs) raised hopes of a wave of new drugs in the Ab category, these hopes were not to be realized for nearly two decades. Mouse-derived MAbs elicited a robust immune response to the mouse protein itself. The consequences of this immune response were twofold: it limited the potency of the MAb by enhancing its clearance rate, and it posed a significant risk of the patient developing anaphylaxis with repeated dosing.

The application of recombinant DNA techniques has enabled replacement of progressively larger portions of mouse MAb with human sequences (Fig. 1). The first significant step was a process for the generation of chimeric MAb, in which the constant domains of both heavy and light chains were replaced with the corresponding human Ig sequences; only the V regions were retained as murine, thereby reducing the mouse protein sequences to approx 33% of the total molecule (3). Of the 14 MAbs approved for market over the past decade, 4 are chimeric. A further refinement of this approach resulted in humanized Ab, in which the framework of the V regions is replaced with human sequences, and only the CDR (approx 5–10% of the total Ab) is retained as murine (4). Six humanized MAbs have received marketing approval.

With continued technological evolution, generation of fully human MAbs is readily achievable. In essence, there are two general approaches. In one, Ab libraries are generated from human immune cells. These libraries generally contain either single-chain Fvs (scFvs) or Fabs displayed on the surface of bacteriophages (5,6) or ribosomes (7), and are collectively termed *Ab display libraries*. Such libraries can be screened using a variety of approaches to select Abs that bind to a particular Ag. While the assembly of a quality display library requires considerable expertise and time, once established, it serves as a renewable source for Abs to a variety of Ags. A significant shortcoming of the display library approach for developing Abs for indications outside of infectious disease, however, is that it is not possible to immunize people with the target Ag. With-

out the normal affinity maturation process associated with repeated immunization, the affinities of Ab derived from display libraries tend to fall short of the range required for therapeutics. As a consequence, further efforts are often required to enhance the affinity of display-derived MABs in vitro. The first fully human Ab to be approved for therapy (in early 2003) was derived from phage-display technology.

The alternative to screening Ab libraries is using transgenic mice that have been genetically engineered to produce human Abs. In the available strains of such mice, the murine heavy chain and  $\kappa$  light chain Ig loci have been functionally inactivated using gene-knockout technology. Human Ig loci have been introduced as transgenes (8,9) or minichromosomes (10). These transgenic mice can be used essentially as normal mice to derive MABs, but the end product is a fully human Ab rather than a mouse Ab. Using standard techniques, it is possible to immunize these mice repeatedly, driving the immune response to generate high-affinity Abs of therapeutic quality through the normal affinity maturation process.

### Antibody Selection

#### CLASS AND ISOTYPE

The IgG class can be subdivided into four isotypes—IgG1, IgG2, IgG3, and IgG4—in humans. The heavy chain alone determines the isotype. The marketed humanized Abs are of either the IgG1 or IgG4 isotype. IgG2 Abs are in clinical development. The IgG3 isotype, although similar to IgG1 in function, has a much shorter plasma half-life than the other Abs, and is thus of less interest as a therapeutic.

Abs of the IgG1 isotype are the most effector-function rich (11). When manufactured with appropriate attention to carbohydrate content, IgG1 Abs are optimal for complement fixation and targeting effector cells through Fc-receptor binding. Therefore, when the objective of Ab therapy is to directly kill the target cell, the isotype of choice is IgG1.

Abs of the IgG2 and IgG4 isotypes are comparatively inactive regarding effector function, with subtle differences between them: IgG2 is the weaker of the two in Fc-receptor binding, and IgG4 is weaker in complement fixation. IgG4 may pose a special challenge, in that the inter-heavy chain disulfide bonds in native IgG4 have been reported to be unstable, resulting in exchange of half-molecules (12). This problem can be corrected with a discrete point mutation in the hinge region (Ser241 to Pro) (13).

#### ANTIBODY FRAGMENTS

A perceived advantage of Abs as therapeutics, in addition to their exquisite specificity, is their long circulating half-life. For most indications, maintaining effective plasma concentrations over a prolonged period of time is desirable. Notably, 13 of the 14 therapeutic Abs currently available are intact Abs.

In certain situations, however, short plasma residence times are desirable, typically when the therapeutic effect can be achieved quickly and when longer exposure to Abs risks normal tissue damage. In such situations, a smaller version of the Ab that retains its Ag-binding properties, but dispenses with the bulky Fc domain, is the preferred drug. Typically, a soluble Fab fragment retains the same binding affinity for a soluble monovalent Ags as the intact molecule, but the avidity (the net effect of an Ab binding two Ag on a cell surface at once) will obviously be decreased. Fabs offer the advantage of being amenable to manufacture in *Escherichia coli*, which can be more economical than using mammalian cell culture production methods.

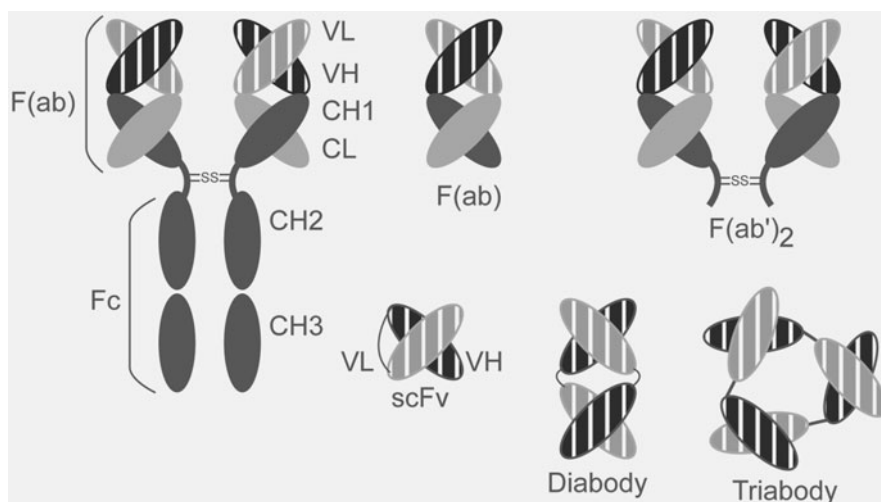


Fig. 2. Structures of intact IgG and engineered antibody fragments.

An alternative to the Fab format is the scFv format. An scFv consists of the  $V_H$  and  $V_L$  domains joined in tandem by a linker peptide, essentially an Ag-binding site free of other Ab domains. Certain Ab-generation technologies involve the use of scFvs throughout, in which case the scFv is the direct product. If the starting format is an intact Ab, however, converting to an scFv format may incur a loss of affinity. Like soluble Fabs, scFvs are suitable for bacterial manufacture.

Other genetically engineered variants of Ab structures are possible and offer advantages for certain applications. Diabodies and triabodies (Fig. 2) are other multivalent structures based on the scFv format.

#### ANTIBODY FUNCTION

Therapeutic Abs can exert their effects through many mechanisms, using a variety of structural components. In some cases, when the desired effect is to block the interaction of two proteins, the important structural attribute is the bulky Ag-binding site. An Ab may confer a specific advantage over a small molecule inhibitor when the ligand's and receptor's binding surfaces are relatively large. In such cases, the only use for the Fc domain is to enhance the plasma half-life. For blocking Ab, the choice of isotype will depend on the nature of the target Ag. If the target Ag is on the cell surface, an IgG4 or IgG2 Ab, both of which are weak modulators of effector functions, would be appropriate. Either isotype may be modified to further reduce its ability to modulate effector function (14–16). If the target Ag is a soluble ligand, an IgG1 may be preferred over an IgG2 or IgG4 to enhance Fc receptor-mediated clearance of the Ag-Ab complexes.

Less commonly, the desired effect, rather than to block binding of a ligand to a receptor, is to mimic ligand binding. While only a limited number of examples of agonist Ab exist (17–20), the established feasibility of this approach offers exciting possibilities for future therapeutic modalities. Of particular interest to oncologists are agonist Abs that target surface molecules capable of delivering signals for apoptosis (e.g., Fas

and the TRAIL receptors). Studies with agonist Abs to date indicate that formation of homodimers is critical.

The most common application of Abs in oncology is the directly mediated killing of tumor cells, which can be accomplished through several approaches. The most straightforward approach from a manufacturing perspective is to rely on the inherent effector function of an IgG1 Ab to harness ADCC and/or CDC for target-cell destruction. Future cytotoxic Abs for cancer therapy will likely take advantage of a series of effector-enhancing mutations in the Fc domain recently identified through a systematic mutational analysis of the entire IgG1 Fc domain (21). An alternative strategy is to use mammalian cell lines for manufacturing that have been engineered so as to enhance the prevalence of certain carbohydrate structures linked to Asn297 that have been correlated with enhanced ADCC activity. Accordingly, Rituxan with enhanced ADCC activity has been produced in a Chinese hamster ovary (CHO) cell line transfected with  $\beta(1,4)$ -*N*-acetyl-glucos-aminyl-transferase (GnT III), the enzyme that adds bisecting GlcNAc residues to complex oligosaccharides (1). The enhanced ADCC activity was shown to be due to higher-affinity binding to Fc $\gamma$ RIII. Production of Abs deficient in fucose from a different CHO cell line has been shown to enhance binding to Fc $\gamma$ RIII (2).

To further enhance an Ab's cytotoxicity, the Ab can be conjugated to toxins or radioisotopes. This approach offers the potential for highly potent immunotherapy; it also risks toxic side effects, either through binding of the Ab conjugates to normal tissues or through accumulation of toxins or radioisotopes in the circulation or tissues. While only three Abs in this category, Mylotarg (conjugated to the toxin calicheamicin), Zevalin (conjugated to Y-90), and Bexxar (conjugated to I-131) have been approved, intense efforts aimed at optimizing conjugation chemistries and selecting appropriate cytolytic agents are certain to yield additional Ab conjugate products in the future.

#### ANTIBODY AFFINITY

The affinity of an Ab for its target Ag is an important consideration when determining the optimal design goals for a therapeutic Ab. In many cases, increasing Ab affinity will increase Ab potency and decrease clinical dose requirements. While no evidence shows that higher affinity for intact Ab is detrimental, there is typically an affinity limit for binding to a particular Ag, beyond which no benefit is added. This affinity limit will be determined largely by the Ag density on the cell surface and the surface residence time subsequent to Ab binding.

### **Clinical Pharmacology**

#### *Pharmacokinetics*

MAbs generally exhibit a favorable pharmacokinetic profile. Pharmacokinetic variability among patients is low, which helps ensure that all patients receiving a given dose achieve appropriate exposure to the drug. Unlike many therapeutic small molecules, Abs are not subject to metabolic drug–drug interactions and are not substrates of the multidrug resistance efflux pumps. Intact Abs have long elimination half-lives, which permit convenient, infrequent dosing schedules. If a short elimination half-life is desirable, Ab fragments may yield the optimum half-life and distributional properties. Like most biologics, Abs must be administered parenterally; however, the pharmacokinetic profile often will support dosing on the same schedule as concurrent chemotherapy regimens.

The catabolic rates of the endogenous human Igs have been well characterized (22). The half-lives for IgA, IgD, IgE, and IgM are approx 5.8, 2.8, 2.5, and 5.1 d, respectively. The half-life of IgG varies according to isotype: 3 wk for IgG1, IgG2, and IgG4; approx 8 d for IgG3. Intact human and humanized therapeutic Abs will usually have half-lives comparable to the corresponding endogenous isotype unless Ag levels are sufficiently high to alter Ab disposition. Murine and chimeric Abs administered to a human usually exhibit more rapid clearance and shorter half-lives than their endogenous counterparts (23). Because most therapeutic Abs are based on IgG, further discussion of Ab pharmacokinetics is limited here to the IgG class.

#### DISTRIBUTION

After iv administration, intact Abs and Ab fragments typically undergo biphasic elimination from serum, beginning with a rapid distributive phase. The calculated volumes of distribution (volume of the central compartment [ $V_c$ ] steady-state volume of distribution [ $V_{ss}$ ]) are similar for intact Abs and Ab fragments. Abs initially distribute into plasma volume and then into limited extracellular space.  $V_c$  is approx equal to plasma volume, with reported values in humans ranging from 2 to 3 L (23).  $V_{ss}$  ranges from approx 3.5 to 7 L (23). Tissue distribution studies in animals using radiolabeled Abs have shown localization predominantly to highly perfused organs such as kidney, spleen, heart, lungs, and liver (24).

Igs have restricted access across diffusional barriers unless transport is facilitated by specific mechanisms. IgG has limited ability to cross the blood-brain barrier; endogenous IgG concentrations in the cerebral spinal fluid are approx 0.1% that of serum (25,26). All IgG subclasses are transported across the placental barrier by the neonatal Fc receptor (FcRn) (27), although the IgG1 isotype is the most efficiently transferred (28,29). FcRn also mediates the vectorial transport of IgG into the lumen of the intestine (30) and lung (31).

Tumor architecture poses a significant barrier to delivery of IgG to tumor targets. Several clinical studies have shown very low tumor uptake of Abs with 0.0001–0.01% of the injected dose localizing per gram of tumor (32). Abs penetrate the tumor by diffusion and convection, but interstitial fluid pressure gradients from the center to the periphery of the tumor hinder movement of Abs into the interstitial space (33–35). Ab binding to the target Ag may pose a further barrier to Ab penetration (36,37). Because of binding-site barriers, the time required to penetrate a tumor may be increased by higher Ab affinity and a longer dissociation half-life of the Ab from the Ag (38). Collectively, diffusional barriers, heterogeneous blood supply, and variable Ag distribution may result in heterogeneous and limited penetration of the Ab into the tumor, which can compromise Ab therapy in patients with large solid tumors and bulky lymphomas (39).

Engineered Ab fragments may exhibit improved tumor penetration, while maintaining the valency and avidity of an intact Ab (39,40). Penetration advantages for these smaller Ab fragments, however, may be largely offset by more rapid clearance and short elimination half-lives (32). Ab fragments may provide an advantage for delivery of immunoconjugates, when more efficient delivery to tumor than normal tissue and rapid clearance from the circulation may be desirable. The pharmacokinetic ability to maintain very high serum concentrations of an intact IgG with high potency and affinity may provide the best solution to low tumor partitioning in the setting of chronic therapy with a naked Ab.

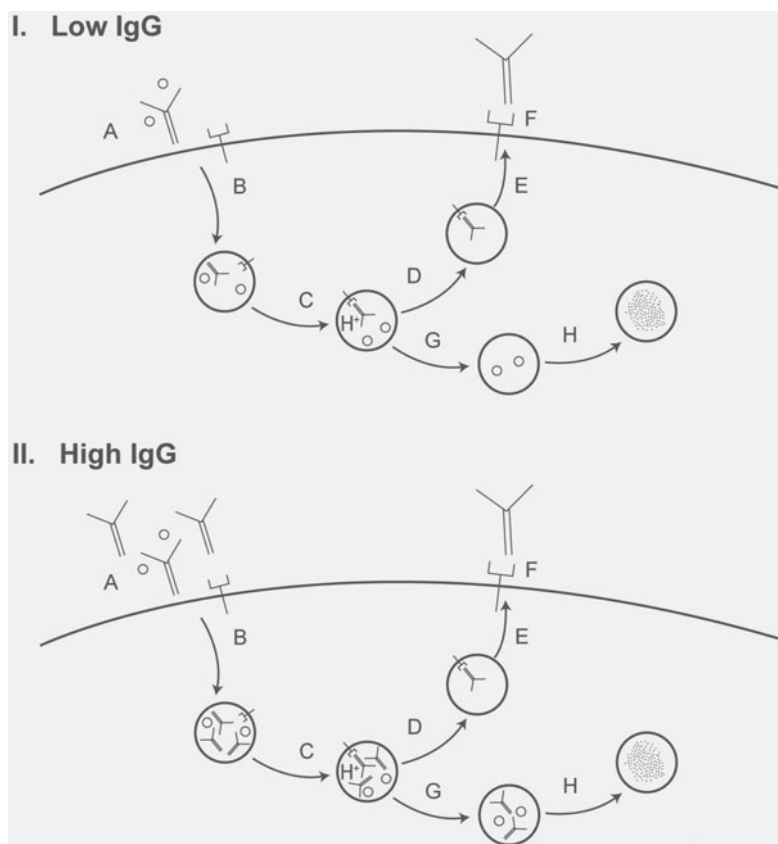


Fig. 3. Mechanism of protection of IgG from catabolism. IgG and plasma proteins (A) are internalized into endosomes of the endothelium (B). In the low pH of the endosome (C), binding of IgG to FcRn is promoted. IgG salvaged by receptors recycles to the cell surface and dissociates in the neutral pH of the extracellular fluid (D, E, F). Unbound proteins are shunted to the lysosomes for degradation (G, H). At low IgG concentrations, FcRn efficiently salvages IgG from catabolism. High IgG concentrations saturate FcRn, and excess IgG is catabolized. (Adapted from refs. 41 and 27.)

## ELIMINATION

The elimination rate of IgG-based therapeutics is regulated by several key factors, including the presence of an intact FcRn-binding domain, isotype, species of origin, presence of an Ag-binding sink, molecular weight, and Ab immunogenicity. Depending on the Ab's properties, elimination half-lives can range from several minutes to several weeks. Rational choice of an intact Ab or an Ab fragment provides considerable flexibility in achieving the optimum pharmacokinetic profile for the intended therapeutic target.

Unless pharmacokinetics are altered by Ag binding or immunogenicity, intact human IgG1, IgG2, and IgG4 Ab will exhibit linear pharmacokinetics and an elimination half-life of approx 3 wk. The extraordinarily long half-life of these Ab isotypes is due to catabolic protection by FcRn, as originally proposed by Brambell (Fig. 3) (27,41).

FcRn is expressed on hepatocytes, endothelial cells, and phagocytic cells of the reticuloendothelial system (42). When IgG undergoes endocytosis, the low pH of the endosome promotes binding of the IgG Fc domain to FcRn, which recycles IgG to the cell surface and salvages IgG from lysosomal degradation, while causing dissociation of Ag. The captured IgG, now stripped of Ag, is protected from lysosomal degradation, recycled to the cell surface, and subsequently released back to the circulation (27). Binding to FcRn is saturable, so more rapid clearance of IgG occurs when total (endogenous plus exogenous) IgG concentrations are high, and slower clearance is observed when total IgG concentrations are low (41,43). With the exception of iv immune globulin therapy, therapeutic doses of Ab are too low to increase total IgG serum concentrations; therefore, pharmacokinetics are usually dose proportional. However, baseline amounts of endogenous IgG may be an important variable explaining interpatient variations in half-life of intact therapeutic Ab (44).

Elimination of intact murine IgG1 and IgG2 Ab is much faster than that of the corresponding human isotypes. Half-lives for murine Abs are in the range of 12–48 h in humans (23). In mice, elimination half-lives are approx 7 d. The comparatively short half-life of murine Abs in humans is owing to low-affinity binding of the murine Fc domain to human FcRn. Human FcRn binds to human, rabbit, and guinea pig IgG, but not significantly to rat, bovine, sheep, or mouse IgG; mouse FcRn binds to IgG from all of these species (45). Chimeric murine Abs have improved FcRn interaction and longer half-lives. Most chimeric Abs have more rapid clearance than human and humanized IgG; reported half-lives have ranged from 3 to 20 d, with most values in the range of 3–9 d (44,46–52).

Ab fragments, including F(ab')<sub>2</sub>, Fab, and scFv, do not retain an FcRn binding domain. Therefore, the half-lives of these fragments are substantially shorter than those of intact IgG, with half-life determined predominantly by their molecular weights. Lower-molecular-weight Fab and scFv fragments are subject to renal clearance, which accelerates elimination (53–55). Reported half-lives have ranged from 11 to 27 h for F(ab')<sub>2</sub> fragments and from 2 to 21 h for Fab fragments (23). The half-life of monovalent and multivalent scFv constructs may range from minutes to several hours (56).

Ag binding can alter the pharmacokinetic profile of Ab. If the Ab binds to an internalized cell membrane Ag, or if the immune complex formed with a secreted Ag is efficiently cleared from circulation, the Ag acts as a “sink” for Ab clearance. In cases in which the immunoassay used to quantitate the Ab does not detect Ab bound to soluble Ag, a short half-life may reflect a decline in free, bioactive Ab concentrations, rather than an irreversible clearance sink. Since Ag binding is saturable, Ag-mediated clearance or assay interference by soluble Ag will result in nonlinear pharmacokinetics, manifested by a short Ab half-life when the dose is below the level that saturates the Ag. At saturating doses, reticuloendothelial system–mediated clearance will predominate and half-life should be similar to that of endogenous IgG. IMC-C225, a chimeric IgG1 Ab against the epidermal growth factor receptor (EGFR), exhibited dose-dependent clearance that was attributed to EGFR binding; saturation of the EGFR sink was used as a surrogate for receptor occupancy and selection of clinical dose (57). Shed Her2 has been reported to affect the pharmacokinetics of trastuzumab, a humanized IgG1 Ab against Her2: in patients with serum Her2 concentrations <500 ng/mL, the average Ab half-life was 9.1 d, whereas in patients with Her2 concentrations >500 ng/mL, the half-life was 1.8 d (58). The half-life of trastuzumab has been reported to be approx 28 d at steady state (59), presumably owing to saturation of the soluble Her2



**Table 1**  
**Affinity of Fc $\gamma$  Receptors for IgG Isotypes and Expression on Blood Cells**

Fc $\gamma$ receptor	Relative affinity for IgG isotypes	Expression on blood cells
Fc $\gamma$ RI	IgG1, IgG3 > IgG4 >> IgG2	M $\phi$ , PMN, DC
Fc $\gamma$ RIIA (R131)	IgG3 > IgG1 >> IgG2, IgG4	M $\phi$ , PMN, PLT
Fc $\gamma$ RIIA (H131)	IgG3 > IgG1, IgG2 >> IgG4	M $\phi$ , PMN, PLT
Fc $\gamma$ RIIB	IgG3 > IgG1 > IgG4 > IgG2	M $\phi$ , B
Fc $\gamma$ RIIIA	IgG1, IgG3 >> IgG2, IgG4	M $\phi$ , NK
Fc $\gamma$ RIIIB	IgG1, IgG3 >> IgG2, IgG4	PMN

M $\phi$ , monocyte/macrophage; PMN, neutrophil; DC, dendritic cell; PLT, platelet; B, B-cell; NK, natural killer cell.

sink; a 3-wk dosing interval has been proposed, rather than the weekly dosing regimen that was originally recommended (60).

Immunogenicity of Ab therapeutics may greatly influence pharmacokinetic profiles. The impact of immunogenicity on safety, pharmacokinetics, and efficacy is discussed in detail later.

### *Mechanism of Action*

MAbs can exert antineoplastic effects through multiple mechanisms that include ADCC/CDC, antiproliferation, apoptosis, antiangiogenesis, inhibition of invasion and metastasis, immunomodulation, and delivery of a radionuclide or immunotoxin to neoplastic cells. Most Abs act through multiple cooperative mechanisms and may exhibit cooperativity with concurrent chemotherapy regimens.

### EFFECTOR FUNCTION

Intact MAbs may kill tumor cells through Fc-mediated effector functions. ADCC is induced by crosslinking Fc $\gamma$  receptors on immune effector cells with MAb-coated tumor cells. CDC results from interaction of tumor-bound MAbs with proteins of the complement system. The ability of an MAb to recruit ADCC and CDC is dependent on the MAbs isotype.

Fc $\gamma$  receptors comprise five classes and subclasses: Fc $\gamma$ RI, Fc $\gamma$ RIIA, Fc $\gamma$ RIIB, Fc $\gamma$ RIIIA, and Fc $\gamma$ RIIIB. Polymorphic variants of Fc $\gamma$ RIIA (131H/R), Fc $\gamma$ RIIIA (48L/H/R and 158V/F), and Fc $\gamma$ RIIIB (NA1 and NA2) have been identified. Polymorphisms in these receptors may have relevance to disease susceptibility (61–65) and response to MAb therapy (66). These receptors are expressed differentially on immune effector cells and differ in their affinity for the IgG isotypes and their potential to elicit ADCC (Table 1) (14). Fc $\gamma$ RI has high affinity for monomeric IgG; Fc $\gamma$ RII and Fc $\gamma$ RIII have low affinity for monomeric IgG and can only interact effectively through high-avidity binding to multimeric complexes or multiple MAb coating a cell surface.

Complement activation by intact MAb may elicit cell killing; CDC is initiated by binding of the complement protein, C1q, to the MAb Fc domain. C1q is multivalent and must bind to multiple MAb molecules attached to a cell to initiate the complement cascade. The ability of IgG to elicit CDC is also isotype dependent: IgG1, IgG3 > IgG2 >> IgG4. The IgG4 isotype has been consistently shown to lack C1q binding and complement activation (14).

## ANTIPROLIFERATION

The ability of tumor cells to produce growth factors and to autostimulate cell proliferation is an essential component of neoplastic cell growth (67). The critical importance of autocrine growth factor and receptor pathways in neoplasia has been recognized since Sporn and Todaro (68) proposed the autocrine hypothesis in 1980. A component of the mechanism of action of two approved MAbs, trastuzumab and rituximab, is through their antiproliferative effects.

Trastuzumab exhibits antiproliferative effects toward several cell lines that express higher than normal levels of *Her2*. In the *Her2*-overexpressing cell line SK-BR-3, trastuzumab reduced the percentage of cells in S-phase and increased the percentage in G0/G1; however, trastuzumab did not affect the MCF-7 cell line that expresses normal amounts of *Her2* (69,70). In SK-BR-3 cells, trastuzumab induced *p27KIP1* and *p130*, consistent with inhibition of cell-cycle progression. The Ab downregulated *Her2* from tumor cell membranes, which may decrease the availability of receptor for homodimerization and heterodimerization (71). In addition to antiproliferative effects, the efficacy of trastuzumab may also be enhanced by antiangiogenic properties and ADCC/CDC (71).

Through binding to CD20 on malignant B-cells, rituximab exerts multiple pharmacologic effects that include cell-cycle arrest, induction of apoptosis, and recruitment of ADCC/CDC (72). The relative contribution of antiproliferative effects to the efficacy of rituximab is unclear, since ADCC/CDC is recognized as a major component of rituximab's efficacy in B-cell lymphomas (66,73).

## APOPTOSIS

Apoptosis is defined as programmed cell death and is typically associated with cellular and nuclear pyknosis, cytoplasmic blebbing, and DNA fragmentation. Apoptosis may be triggered by activation of receptors of the tumor necrosis factor receptor (TNFR) family, most notably TNFR1, Fas, and the receptors for TRAIL, all of which contain cytoplasmic death domains. Ab targeting members of this family may be used in two distinct modes—as agonists to trigger apoptosis of cancer cells, or as antagonists to inhibit tumor cell-induced apoptosis of receptor-bearing tumor-reactive lymphocytes.

Apoptosis can be induced as the result of extensive crosslinking of receptors that normally mediate cell proliferation and differentiation. Accordingly, Abs to CD19 (74), CD20 (75), CD44 (76), major histocompatibility complex class II (77), and EGFR (78) will cause apoptosis when crosslinked with secondary Ab. In vivo, secondary crosslinking of surface-bound Ab may occur by binding to Fc receptors on accessory cells, such as macrophages and neutrophils. The requirement for extensive receptor crosslinking to induce apoptosis may lead to alternative designs for therapeutic Abs in the future to take greater advantage of this mechanism for cell killing (79).

## ANTIANGIOGENESIS

Angiogenesis, the growth of new blood vessels, is an important component of embryonic development. It occurs only infrequently and transiently in adults, particularly during the female reproductive cycle, wound healing, inflammation, and hair growth. Thirty years ago, Judah Folkman (80) observed that tumors depend on angiogenesis for their continued growth and survival. Formation of new blood vessels is necessary to support the increasing tissue mass of a metastatic tumor. Further, because tumor vascu-

lature is inherently unstable, angiogenesis inhibitors can cause regression of established tumors as well (81).

Angiogenesis involves the coordination of invasion, proliferation, differentiation, and regression of new blood vessels. These processes, in turn, involve interactions among a host of extracellular molecules, including growth factors, adhesion molecules, receptors, chemokines, and proteolytic enzymes. Ab targeting members of each of these families are in clinical trials. Identification of new players in angiogenesis has far outstripped the pace of development of new therapeutics. Since an Ab to vascular endothelial growth factor (VEGF) first entered the clinic, four more members of the VEGF family (VEGF-B through VEGF-E) have been identified. Similarly, for years attention was focused on determining the relative functionalities of VEGFR1 (Flt-1) and VEGFR2 (KDR), but recently a VEGFR3 was identified (82).

#### INHIBITION OF INVASION AND METASTASIS

Currently, no Ab therapeutics are approved that act principally through inhibiting invasion and metastasis. A fully human Ab, ABX-MA1, that binds to MCAM/MUC18 and may inhibit metastasis and invasion of human melanoma cells, is in phase 1 clinical study. In preclinical studies, ABX-MA1 has suppressed experimental lung metastasis of A375SM and WM2664 cells and inhibits the ability of the cells to attach to HUVEC cells in vitro; the Ab inhibits the activity of matrix metalloproteinase-2 (MMP-2) in vitro and decreases invasion through Matrigel-coated filters (83). Inhibition of invasion and metastasis may enhance the activity of anti-EGFR Ab. The anti-EGFR Ab C225 downregulated MMP-9 and reduced metastases to lung and lymph nodes in an orthotopic bladder cancer xenograft model (84).

#### IMMUNOMODULATION

MAbs are in development that exert immunomodulatory functions and may stimulate immune responses against tumor cells. Fully human Abs against cytotoxic T-lymphocyte antigen-4 (CTLA-4) are in clinical trials. In preclinical studies, Abs against CTLA-4 have shown promising activity as agents for cancer immunotherapy (85). In another immunomodulatory approach, idiotype vaccines have been used to elicit anti-idiotypic immune responses against tumor-specific Igs in patients with B-cell lymphomas (86).

#### TARGETED DELIVERY OF RADIONUCLIDES AND CYTOTOXINS

MAbs can selectively deliver radionuclides and toxins to cancer cells. For radionuclide delivery, Abs ideally should target noninternalizing, tumor-selective Ag; high-energy  $\beta$ -particle emitters such as I-131 or Y-90 are usually chosen because the long path length of the  $\beta$ -particle can kill macroscopic tumor masses, including regions that are inaccessible to the Ab. For single-cell killing, targeting of micrometastases, or vascular-targeted radiotherapy, use of  $\alpha$ -particle emitters has been proposed to minimize normal tissue toxicity (87,88). Immunotoxin conjugates should be directed against internalizing, tumor-selective Ag using a labile linker that releases a highly potent toxin intracellularly after internalization (89,90). The half-life for linker stability in vivo should not be shorter than the half-life of the Ab carrier; otherwise, selective delivery could be lost due to accumulation of unconjugated Ab and binding competition with the immunotoxin.

Ibritumomab tiuxetan received marketing approval for radioimmunotherapy of patients with relapsed or refractory low-grade, follicular, or transformed B-cell non-

Hodgkin's lymphoma (NHL) (91). The Ab delivers yttrium-90 to the noninternalizing CD20 Ag, which is expressed on malignant and normal B-cells, but not plasma cells. Gemtuzumab ozogamicin received marketing approval for treatment of patients with CD33-positive acute myeloid leukemia (AML) in first relapse, who are not considered candidates for other types of cytotoxic chemotherapy (92). The Ab targets the internalizing CD33 Ag expressed on leukemic blasts and delivers a potent antibiotic, calicheamicin intracellularly. Calicheamicin produces cell death as a consequence of site-specific, double-strand DNA breaks (93,94).

### Safety

MAbs are highly specific for the target Ag. Few Ags, however, are tumor specific, and toxicity may result from interaction with Ag expressed on normal tissue or from the pharmacologic effects of neutralizing a biologically active secreted protein. Non-specific toxicity may occur, including infusion reactions, cytokine release, and hypersensitivity. Immunoconjugates may exert toxic effects on normal tissues through targeting of normal tissue, bystander effects, or catabolism and release of an immunoconjugate. Normal tissue toxicity after treatment with Ab therapeutics may be anticipated, in some instances, based on the expression profile of the Ag and the functional role of the Ag in that tissue.

Cardiac toxicity has been observed after treatment with trastuzumab as a monotherapy and in combination with paclitaxel and anthracyclines. Signs and symptoms include tachycardia, palpitations, exertional dyspnea, and congestive heart failure (95). The incidence of symptomatic heart failure in pivotal clinical trials was 6.0–8.8% as a monotherapy, 8.8–11.0% in combination with paclitaxel, and 26.0–28.0% in combination with anthracyclines (96). In the monotherapy setting, all patients developing heart failure had received prior anthracycline therapy or had a history of cardiac disease. The mechanism of cardiac toxicity has not been fully elucidated but may be related to the functional role of *erbB2* for muscle spindle maintenance and survival of myoblasts (97–99). Additionally, the role of trastuzumab's IgG1 isotype and effects of ADCC and CDC on *erbB2*-expressing cardiomyocytes are unknown.

Abs directed against EGFR have exhibited a dose-dependent skin rash related to EGFR inhibition in skin. The toxicity is manifested as sterile, suppurative folliculitis, most frequently present on the face, scalp, chest, and upper back (100,101). The toxicity has not been dose limiting, and the incidence and severity of this target-related toxicity has been proposed as a biomarker for EGFR saturation and selection of clinical dose (102).

### INFUSION REACTIONS, CYTOKINE RELEASE, AND HYPERSENSITIVITY

MAbs may produce infusion and hypersensitivity reactions. The reactions may be idiosyncratic or related to the target, Ab isotype, crosslinking of target-expressing cells and effector cells, or the immunogenicity of the Ab.

First-dose infusion reactions associated with elevation of serum interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interferon- $\gamma$ , have been observed with many murine, chimeric, and humanized Abs. The cytokine-release syndromes may be mild to life threatening, clinically characterized by fever, chills, rash, nausea, myalgias, and/or hypotension; in rare cases the infusion reactions have been lethal. Mild-to-moderate infusion reactions are commonly observed after administration of trastuzumab. Serious and fatal infusion reactions have been rare; in postmarketing surveillance before

March 2000, 74 worldwide cases, including 9 fatal cases, occurred in 25,000 patients treated with trastuzumab (96). Most of the serious reactions occurred within 2 h after starting the first infusion. Infusion reactions are commonly observed after infusion of rituximab and have been particularly severe in patients with chronic lymphocytic leukemia (CLL) with increased lymphocyte counts (103).

Multiple mechanisms may contribute to first-dose cytokine release. Association of increased lymphocyte counts with severity of reactions after rituximab treatment suggests that the toxicity may be directly related to Ab binding to CD20 on B-lymphocytes (103). Cytokine release after treatment with murine OKT3 is believed to be caused by T-cell opsonization and subsequent lympholysis (104). Crosslinking of effector cells may be a contributing factor, since bispecific Abs targeting Fc $\gamma$ RI and Fc $\gamma$ RIII have been commonly associated with infusion reactions (105,106). Ab isotype may also be important. In a mouse model, infusion reactions were associated with murine IgG1, IgG2a, and IgG2b isotypes, but not IgG3 (107). The chimeric IgG1 anti-EGFR Ab IMC-C225 has caused infusion reactions in patients with cancer, but the fully human IgG2 anti-EGFR Ab ABX-EGF to date has not (100,108,109).

Hypersensitivity reactions related to the immunogenicity of murine, chimeric, and humanized Abs may occur after repeated dosing. The impact of Ab immunogenicity is discussed further.

#### IMMUNOCONJUGATE TOXICITY

Toxicity related to the radionuclide or toxin moieties of conjugated Ab occurs due to effects on neighboring normal cells, delivery of the immunoconjugate to Ag expressed on normal tissues, recycling of the immunoconjugate by FcRn on endothelial cells, or catabolism and release of the conjugate. Ibritumomab tiuxetan specifically targets CD20 but produces severe thrombocytopenia and neutropenia with a median 22- to 35-d duration of cytopenia in most patients receiving the yttrium-90 radioconjugate (110–112). The toxicity is related to the effects of  $\beta$ -emissions on neighboring hematopoietic cells, and ibritumomab tiuxetan is not recommended for use in patients with >25% lymphoma marrow involvement, impaired marrow reserve, hypocellular bone marrow, or with a history of failed stem cell collection (110–112). Gemtuzumab ozogamicin, a calicheamicin immunotoxin conjugate, binds specifically to CD33, which is expressed on leukemic blasts in >80% of patients with AML (92). The immunotoxin produces target-related hematopoietic toxicity, owing to expression of CD33 on myeloid progenitor cells (92). The immunotoxin has been associated with severe and potentially fatal hepatotoxicity, including venoocclusive disease. The mechanism of hepatotoxicity is unknown; however, the liver is a site for catabolism of Igs and metabolism of small molecules, so the toxicity may be related to nonspecific disposition and catabolism of the Ab and toxin.

#### Immunogenicity

Development of an immune response to a therapeutic Ab can negatively impact the Ab's safety, pharmacokinetic, and efficacy profiles. Development of an anti-Ab response may occur from 1 wk to several months after initiation of dosing (113,114). The high immunogenicity of murine Abs significantly impaired their development as therapeutic agents; few instances exist in which induction of a human antimouse Ab (HAMA) response has not precluded the successful development of the Ab therapy (39,115). There are two notable exceptions: ibritumomab tiuxetan has been approved

**Table 2**  
**Immunogenicity of Licensed MABs**

Murine ( <i>n</i> = 6)
Intact Abs: 55% <80%
Fab or F(ab') <sub>2</sub> fragments: <1–8%
Chimeric ( <i>n</i> = 4) (3 intact Abs, 1 Fab fragment)
<1–13%
Humanized ( <i>n</i> = 3), intact Abs
<1–8%

Adapted from ref. 116.

and tositumomab has been recently approved in the United States for treatment of advanced NHL; the immunogenicity of these murine Abs is attenuated by B-lymphocyte depletion and myelosuppression. Human anti-Ab responses are usually designated according to the therapeutic Ab construct: HAMA; human antichimeric Ab (HACA); and human antihuman Ab (HAHA). A HACA response against the murine portion of a chimeric Ab may be referred to as a HAMA response to provide more information about the antigenic determinant. However, an Ab response against the murine portion of humanized Ab is usually designated as a HAHA response. Anti-Ab responses can develop against the toxin moiety of an immunoconjugate, e.g., anti-*Pseudomonas* exotoxin, and are designated as human antitoxin Ab (HATA) or human antidrug antibodies.

An anti-Ab response may be anti-isotypic or anti-idiotypic. An anti-isotypic response is directed against the constant regions of the heavy or light chains and might not directly impair Ag binding, although pharmacokinetic effects may render the interaction functionally neutralizing. Antimouse responses are usually anti-isotypic (116). Anti-idiotypic Abs develop against Ab-specific structural features (idiotopes) of the Ab variable region. An anti-idiotypic response against an idiotope of the CDR should block Ag binding; in principle, a framework-related anti-idiotypic Ab might not be neutralizing (117).

The incidences of Ab immunogenicity for licensed MABs (as of the year 2000), including diagnostic Abs, have been reported and are summarized in Table 2 (116). The incidences for murine fragments, chimeric, and humanized Abs are similar for the products that have successfully reached licensure. The reported incidence for licensed products, however, would not reflect the large number of product failures during drug development related to the immunogenicity of murine and chimeric Abs, and thus underestimates the incidence of immunogenicity during clinical development. A number of the licensed products are administered to immunosuppressed patients.

IMPACT ON SAFETY, PHARMACOKINETICS, AND EFFICACY

Serious safety risks are associated with immunogenicity. Adverse reactions may be local or systemic and may vary from mild injection site reactions to life-threatening anaphylactic and other hypersensitivity reactions (116). The timing of hypersensitivity reactions in relation to an anti-Ab response may be variable. For a humanized anti-A33 Ab, infusion-related adverse reactions were associated with late-onset HAHA responses, but not with early HAHA responses (114).

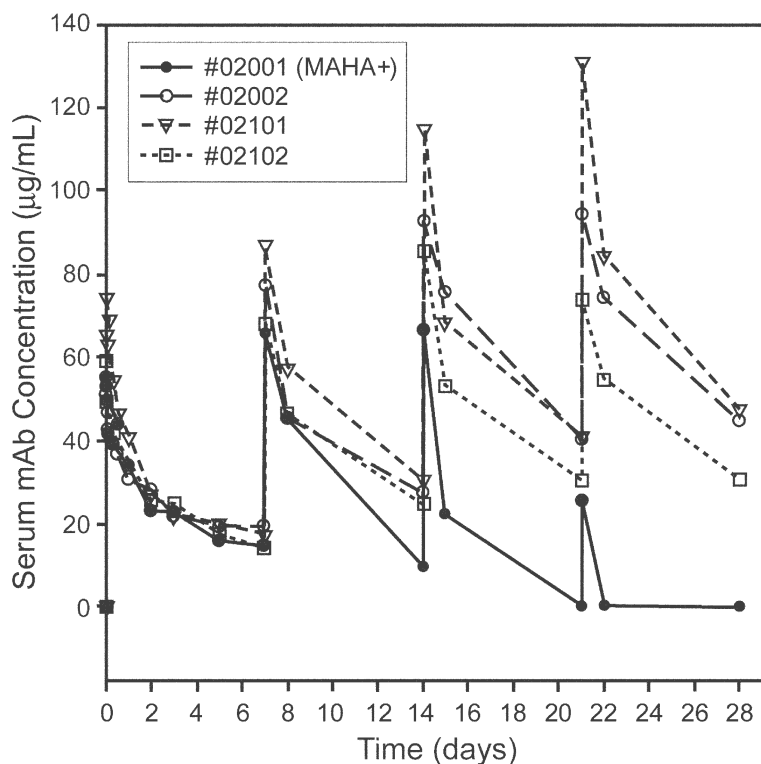


Fig. 4. Impact of MAHA development on antibody pharmacokinetics. Decreasing serum levels of a human mAb followed four consecutive weekly 2.5 mg/kg doses (d 0, 7, 14, and 21) in a cynomolgus monkey developing a positive MAHA response (animal #02001, closed symbols/solid line) relative to monkeys that were MAHA negative (open symbols/dashed lines).

Ab responses against Ab products usually alter pharmacokinetic profiles. A polyclonal response against an mAb will frequently result in immune complex formation that accelerates clearance and impairs targeting of the therapeutic Ab (39). Accelerated clearance has been reported for IMC-C225, a chimeric Ab against EGFR, after development of neutralizing Ab in patients with cancer (118). Development of a HAMA response may lead to rapid clearance of immunotoxin conjugates (119).

Accelerated clearance of therapeutic Ab or neutralization of the Ag-combining site can result in loss of product efficacy, impaired Ag targeting, or interference with Ab-based diagnostic tests (116,120). Loss of efficacy of OKT3 has been observed when HAMA titers are >1:1000 (116).

Because of the sensitivity of pharmacokinetic profiles to the development of anti-Ab responses, pharmacokinetics should be monitored in parallel with immunogenicity assays to determine the incidence and consequence of product immunogenicity. Pharmacokinetic immunoassays should ideally be established using Ag or a neutralizing anti-idiotypic Ab as the capture reagent; such a format will be sensitive to the development of neutralizing Ab responses that block binding to Ag, in addition to anti-Ab responses that enhance clearance. An example of pharmacokinetic correlation with the results of a monkey-antihuman Ab (MAHA) immunogenicity assay is shown in Fig. 4 for a human IgG2 Ab administered to cynomolgus monkeys.

## HUMANIZED AND FULLY HUMAN ANTIBODIES

The development of Ab humanization technology has successfully decreased the incidence of immunogenicity observed with murine and chimeric MAbs (121). However, because of the residual murine protein (up to 10%) in humanized Abs, immunogenicity concerns have not been eliminated. For example, a humanized Ab against A33 produced a 66% incidence of HAHA responses in phase 1 and 2 clinical studies (114).

Whether the development of fully human Ab technology will further reduce or eliminate immunogenicity remains controversial. In theory, all Abs are potentially inherently immunogenic, whether or not they are human Abs, because of the uniqueness of the Ab idiotopes (116). The hypothesis that all Abs are immunogenic may be traced to the anti-idiotypic network theory of Niels Jerne (122). In immune network theory, the existence of an idiotypic–anti-idiotypic network of interactions serves as a regulatory mechanism for immune responses. Nevertheless, hypotheses regarding the inherent immunogenicity of human Abs have not been supported by clinical studies of human Abs as idiotypic-specific vaccines in B-cell lymphomas. To the contrary, human Abs have proven to be extremely poor immunogens in humans, requiring the use of adjuvants and fusion proteins to elicit an immune response (123,124).

Early clinical studies of fully human Abs derived from transgenic XenoMouse® technology, to date, have supported that fully human Abs derived from *in vivo* sources are nonimmunogenic. In phase 1 and 2 clinical trials of ABX-IL8, a fully human Ab to human IL-8, and ABX-EGF, a fully human Ab to EGFR, no HAHA responses or alterations of pharmacokinetic profiles have been observed after chronic dosing (108,109,125).

While human Abs themselves may be well tolerated, immunogenic human Abs theoretically could be produced by phage display technologies. Phage display Abs are generated as the result of random combinations of heavy and light chains and are selected *in vitro*. The panning process imparts additional inadvertent selections such as compatibility with the phage lytic process. In addition, random pairing of heavy and light chains and mutations that are subsequently engineered to improve affinity have not undergone the *in vivo* selection process that would potentially eliminate Abs with unusual conformations or amino acid changes. While fully human in origin, human Abs generated by phage display may have a greater potential for being immunogenic compared with Abs obtained directly from *in vivo* sources. Consistent with this possibility, a 12% incidence of neutralizing HAHA responses has been reported for patients with rheumatoid arthritis receiving monotherapy with the phage display–derived human MAb, adalimumab (126).

## MAb Therapy for Cancer: Clinical Status

MAb therapy has assumed increasing importance over recent years in treating malignancies (127). Four MAb therapeutics are approved for the treatment of hematologic malignancies in the United States: three for the treatment of lymphoma, and one for the treatment of AML. One MAb product, trastuzumab, has been approved to treat solid tumors, specifically advanced breast cancer (92,128–130). Several MAbs for treating cancer are in late-stage clinical development. Additionally, many Abs to various cancer targets are in early stage clinical development. This review focuses mainly on approved MAbs and those now in late-stage clinical development.



## **Hematologic Malignancies**

Phenotypic characterization of leukemias and lymphomas has been standard for more than a decade to help characterize these diseases. Therapeutic MABs have been designed to specifically target such cell-surface Ags.

### **Naked Antibodies**

#### **RITUXIMAB**

In 1997, the Food and Drug Administration (FDA) approved the first MAB for the treatment of cancer in the United States (128). Rituximab, a chimeric MAB to CD20, was approved as a single-agent therapy for patients with relapsed or refractory low-grade malignant NHL, with a recommended dose of 375 mg/m<sup>2</sup> administered intravenously once a week for a total of four infusions (128).

CD20 is an epitope exclusively expressed on normal and malignant B-lymphocytes, but not on stem cells or plasma cells (111). As a result of this expression pattern, Ig can be produced by plasma cells continuously and depleted B-cell populations replenished by intact stem cells (131). CD20 is not modified or endocytosed after binding with Ab, and it is not shed from the cell membrane after Ab ligation (131,132). Several mechanisms are believed to be involved in the activity of rituximab, including ADCC, CDC, and apoptosis induction; however, the predominant mechanism of activity appears to be ADCC (111,132–134).

Pharmacokinetic studies have revealed that the mean maximum plasma concentration ( $C_{\max}$ ) and mean terminal half-life ( $t_{1/2}$ ) of rituximab increase linearly with each subsequent infusion and are greater in patients who respond to treatment with rituximab than those who do not (128,131,135,136). An analysis of 166 patients treated with the recommended regimen of rituximab showed that the mean half-life increased and mean clearance rate decreased over the 3-wk treatment period. Rituximab was detected up to 6 months after treatment in some patients, probably as a result of depleted circulating CD20-positive B-cells and saturation of involved lymph nodes (131).

Rituximab was studied initially in patients with relapsed or refractory low-grade malignant NHL (111,132,133). An overall response rate of 48% with a median response duration of 13 months was observed in a pivotal study in patients with low-grade malignant lymphoma (132). Subsequent studies of rituximab in other B-cell lymphomas resulted in response rates ranging from 14% in small lymphocytic lymphoma to 46% in follicular lymphoma (132).

Some patients are resistant to rituximab treatment (137). Potential explanations for this apparent resistance include the known effects of chemotherapeutic agents on immune effector mechanisms and decreased CD20 expression, leading to decreased binding of the Ab to the tumor cell (137). Forty to 50% of previous responders benefit from a second course of treatment, with many experiencing a longer remission with the second course (137).

As a result of its efficacy as a single-agent therapy for NHL, rituximab has been investigated in combination with chemotherapeutic agents. When patients with previously untreated, aggressive B-cell NHL were treated with cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) in combination with rituximab, an overall response rate of 94% was observed, with 61% of patients achieving a complete response (133). In a randomized study of the GELA group in France, CHOP in combination with rituximab also resulted in a significantly greater response rate in patients with diffuse

large B-cell lymphoma (DLCL) than in patients treated with CHOP alone (76% vs 63%) (138). Furthermore, significant differences were observed between the two treatment groups for event-free survival at 2 yr (57% vs 38%, respectively) and overall survival at 2 yr (70% vs 57%, respectively) (138).

Rituximab has also shown efficacy in patients with low-grade or follicular B-cell lymphoma treated in combination with fludarabine (139). Specifically, 93% of patients responded with a median duration >14 mo, and 89% had nodal response (139).

Rituximab is generally well tolerated, regardless of whether it is administered as a single agent or as part of a combination therapy (111,128,135–137,140–143). In studies of rituximab administered as a single agent, infusion-related reactions, including hypotension, fever, and rigors, were observed primarily with only the first dose and usually occurred within 2 hours of dosing (132). Patients treated with rituximab and CHOP experienced no significant differences in hematologic toxicities compared with patients treated with CHOP alone, and, as with single-agent rituximab therapy, patients commonly experienced infusion-related reactions with the first dose (133). When rituximab was combined with fludarabine, hematologic toxicities, such as grade 4 neutropenia, required a 40% reduction in the standard dose of fludarabine and prophylactic trimethoprim-sulfamethoxazole (139). Some patients treated with this combination therapy developed herpes zoster infections; therefore, prophylactic treatment with acyclovir is recommended for patients treated with this combination (139). No HAMA responses have been observed, and only 1% of the patients have had a detectable HACA response (144).

Approximately 36,000 patients received rituximab from the date of its approval in 1997 through May 1999 (144). During this time, the most common side effects were infusion related and were manageable with supportive care (131). Fewer than 1% of patients experienced serious cardiopulmonary reactions resulting in death or tumor lysis syndrome within 12–24 hours after dosing (144). Additionally, serious late effects, including arthralgia, bullous cutaneous reactions, cytopenias, rashes suggesting serum sickness, vasculitis, and uveitis were reported up to several months after treatment with rituximab (136).

In summary, rituximab has shown activity in patients with hematologic malignancies, including follicular lymphoma, DLCL, mantle cell lymphoma, and CLL, as well as plasma cell disorders, including posttransplant lymphoproliferative disorder and Waldenström's macroglobulinemia (112,135,142,145–147). Other indications being investigated or in the planning stages include multiple myeloma, Hodgkin's disease, CD20-positive leukemias, immune thrombocytopenic purpura, autoimmune hemolytic anemia, IgM polyneuropathies, rheumatoid arthritis, lupus, and other vasculitis syndromes (111,141).

#### ALEMTUZUMAB

Alemtuzumab, a humanized anti-CD52 Ab, has been investigated for the treatment of lymphoid malignancies (127,148,149). Hematologic malignancies were initially targeted because alemtuzumab clears lymphocytes from peripheral blood rapidly, clears lymphocytes from bone marrow at a slower rate, results in very few cases of tumor lysis syndrome, and rarely induces a response in lymph nodes (148). Therefore, CLL was the target indication for alemtuzumab (127,148). The US FDA approved alemtuzumab in May 2001 as a single-agent therapy for patients with B-CLL who have failed prior chemotherapy (129).

CD52 is a nonmodulating Ag that is highly expressed on normal and malignant mature lymphocytes of T- and B-cell lineage; thus, alemtuzumab binds to normal and tumor cells expressing CD52 and can cause profound lymphopenia (148). However, CD52 is not expressed on hemopoietic stem cells; thus, normal cell loss is expected to be transient because normal cells can regenerate from normal stem cells (127,148). The mechanisms of action of alemtuzumab appear to be ADCC and CDC (148).

During the early development of alemtuzumab, two patients with NHL achieved responses. They had marked reduction of lymphoma cells in the blood and bone marrow with decreased splenomegaly, and lymphadenopathy resolved in one patient (127).

Clinical studies of alemtuzumab in patients with CLL have resulted in response rates ranging from 20% in patients with refractory disease to 89% in patients without previous therapy (127,148,149). Response rates ranging from 67 to 75% were observed in three clinical studies in which 36 patients with refractory T-cell prolymphocytic leukemia were treated with alemtuzumab (127). In one study, 60% of patients with T-cell prolymphocytic leukemia treated with alemtuzumab achieved a complete response compared with 10% of patients treated with deoxycoformycin (148). Although alemtuzumab is not curative for the disease, as evidenced by the short-lived mean survival time of 9 mo, additional remissions have been achieved with subsequent courses of alemtuzumab (127,148,149).

Patients treated with alemtuzumab by iv infusion have experienced fever, rigors, and nausea occurring primarily with the first dose due to the release of TNF- $\alpha$  and IL-6; however, these side effects can be reduced by administering alemtuzumab subcutaneously. The most significant side effect of alemtuzumab is profound and persistent lymphopenia (127,148). An increased rate of opportunistic infections during the first 6–8 wk of treatment with alemtuzumab has been observed in heavily pretreated patients, particularly in patients previously treated with fludarabine, but this can be managed with acyclovir, cotrimoxazole, or itraconazole prophylaxis and close observation (127,148).

#### EPRATUZUMAB

Epratuzumab is a humanized MAb designed to target the CD22 Ab in B-cell malignancies (150). CD22 is an adhesion molecule found exclusively on B-lymphocytes, and it is involved in the activation, proliferation, and death of B cells, as well as the interaction between B and T cells (150).

In a phase 1 to 2 clinical study, 24 patients with relapsed or refractory DLCL were treated with epratuzumab (111). Of the 17 evaluable patients, 11% achieved a complete response and 18% achieved a partial response, with responses lasting from 3 to >24 mo. Currently, epratuzumab is being evaluated in patients with relapsed or refractory NHL.

#### ANTIBODIES TO CD19

CD19 is a tumor-associated Ag that is almost exclusively expressed on B-lineage lymphocytes. It is found in several hematologic malignancies, including B-ALL, CLL, hairy-cell leukemia, Burkitt's lymphoma, and other B-cell NHLs, but not in chronic myelogenous leukemia, AML, or T-cell acute lymphocytic leukemia (T-ALL) (151,152). Because of this expression pattern, CD19 is useful in the differential diagnosis of B-cell vs T-cell ALL and other leukemias and lymphomas (152). Furthermore, because CD19 is not usually lost during neoplastic transformation, B-lineage

neoplasms are likely candidates for immunotherapy targeting CD19 (152). Finally, because hematopoietic stem cells do not express CD19, they are not targeted and can repopulate B-lineage cells after treatment (152).

While the primary focus of anti-CD19 treatment has been on toxins conjugated to anti-CD19 MAb, purging of neoplastic cells in harvested bone marrow has also been studied. Bone marrow is harvested from patients and treated with anti-CD19 Ab that purges the tumor cells by complement-mediated lysis, magnetic bead depletion, or immunotoxin-mediated killing, and then the tumor cell-free bone marrow is reintroduced into patients (152). While the complement-mediated lysis approach depleted neoplastic cells without affecting hemopoietic cells, the magnetic bead approach removed residual neoplastic cells and was more effective overall (152). Immunotoxin-mediated killing has also been effective. CD19 cells were depleted from normal bone marrow containing CD19-positive B-ALL cells treated with anti-CD19 immunotoxin and mafosfamide (152).

### *Immunotoxins*

Generally, the efficacy of conjugated MAb is dependent on several factors, including the stability of the linker coupling toxin to Ab; the ability of MAb to specifically target tumor cells; the intracellular trafficking, uptake, and tissue distribution patterns of the conjugated Ab; and the inherent sensitivity of the targeted tumor cells to the toxin (90,153,154). Therefore, the effectiveness of these therapies is both target and toxin dependent. The use of immunotoxin has been limited because of unacceptable side effects potentially caused by limited uptake by the tumor cell and bulk uptake by Fc receptors expressed in normal organs, such as the liver, spleen, and lung, in addition to the immunogenicity inherent in some toxins (90;153;154).

### IMMUNOTOXINS TARGETING CD19

Toxin-conjugated anti-CD19 Abs have been evaluated. Initial clinical studies of an anti-CD19/ricin A conjugate in patients with NHL indicated that the treatment induced responses (complete, partial, and transient); however, patients also experienced hepatotoxicity, HAMA responses, and rapid Ab clearance (152). A clinical study of an anti-CD19 MAb conjugated with deglycosylated ricin A toxin (HD37-dgA) was conducted in eight patients with NHL (155). Although all patients experienced disease progression within 1 mo of treatment, nodes were reduced during treatment in three of the five patients who had palpable nodes at study entry (155). Toxicities included capillary-leak syndrome with hypoalbuminemia, orthostatic hypotension, and weight gain, all of which were dose dependent; and diarrhea, fever, nausea, and vomiting (155). More important, hepatotoxicity, an associated effect of immunotoxins, was not observed, and plasma concentrations were maintained for up to 36 h (155). Two patients developed a human antiimmunotoxin Ab response (155).

### GEMTUZUMAB OZOGAMICIN

Gemtuzumab ozogamicin is a humanized anti-CD33 MAb of the IgG4 isotype conjugated to calicheamicin, a highly potent antitumor antibiotic that cleaves double-stranded DNA (92). This conjugated MAb was approved for treating CD33<sup>+</sup> AML in first relapse in elderly patients (92,129).

CD33 is expressed on myelomonocytic progenitors and precursors, monocytes, and myeloid dendritic cells (92). The Ag is internalized after binding with Ab, and is

expressed on leukemic blast cells in >80% of patients with AML (92). Additionally, because CD33 is expressed on myeloid progenitor cells, but not on hematopoietic stem cells or nonhematopoietic tissues, bone marrow suppression following depletion of CD33 positive cells is transient (92,129).

Phase 2 studies in patients with AML in first relapse showed that gemtuzumab ozogamicin induced remissions in 30% of patients; the duration of remissions averaged 2 mo, but was increased to >18 mo in patients receiving subsequent hematopoietic stem cell transplants (92).

Associated toxicities and known side effects include neutropenia, thrombocytopenia, increased hepatic enzymes, increased bilirubin, and postinfusion fever and chills (92). Hypersensitivity reactions, including infusion reactions, anaphylaxis, and pulmonary events have occurred; in rare cases, these events have been fatal (156). Severe, and in some cases fatal, hepatic venoocclusive disease has been observed in three clinical studies with gemtuzumab ozogamicin. Specifically, 4% of patients experienced ascites, weight gain, and increased bilirubin, indicating venoocclusive disease; three of 36 (8%) patients in one study, but <1% overall, died of venoocclusive-like disease (92). Two patients in a phase 1 study developed an HATA response against calicheamycin (156).

#### ANTI-CD22 *PSEUDOMONAS* EXOTOXIN

BL22 is a recombinant immunotoxin composed of the variable domain (Fv) of the anti-CD22 MAb, RFB4; and PE38, a fragment of the *Pseudomonas* exotoxin (157). Because CD22 is rapidly internalized and modulated after binding with Ab and reexpressed on the cell surface, it is an ideal target for immunotoxins (150). PE38 is a smaller molecule than the deglycosylated ricin A chain commonly used in immunotoxins and contains the domains responsible for cell death, but not cell binding (157).

CD22 is expressed in all patients with hairy cell leukemia (157). A clinical study of BL22 in 16 patients with hairy cell leukemia who had inadequate responses to previous cladribine therapy showed that 13 of 16 (81%) patients achieved a response; most achieved a complete response (157,158). Three patients relapsed during the follow-up period but achieved another complete response after being retreated with BL22 (157). Serious, but reversible, hemolytic uremic syndrome was reported in 2 of 16 (13%) patients during treatment (157). Less serious and transient side effects included hypoalbuminemia, increased aminotransferase concentrations, slightly increased creatinine concentrations, nausea, myalgia, and edema (157). No vascular-leak syndrome was observed in the patients treated with BL22 (157). Of the 16 patients, 4 (25%) had an immune response with neutralizing Ab production.

#### Radioimmunotoxins

Radioisotope-conjugated MAbs are expected to enhance the tumoricidal effects of the Ab in treating lymphoid malignancies and other tumors (159). Furthermore, radiation can also reach and kill tumor cells in the vicinity of radioimmunotoxin-bound cells; thus, bulky or poorly vascularized tumors may be more effectively targeted and treated than with naked Ab alone (159,160). However, radioimmunoconjugates can kill or damage normal cells, including hematologic tissues, and potentially resulting in toxicity (110).

The two most commonly used isotopes for radioimmunotherapy are I-131 and Y-90, both of which emit  $\beta$  particles that can span several cell diameters, effectively killing

Ab-bound tumor cells and nearby tumor cells not bound by the Ab (159). I-131 has a half-life of 193 h, does not accumulate in bone, and can be blocked from the thyroid by potassium iodide (161). Y-90 has a shorter half-life of 64 h, which permits outpatient treatment (112). Additionally, Y-90 has a greater radiation penetration (5–10 vs 1 mm) and theoretically provides advantages in treating bulky lymphomas (112).

#### IBRITUMOMAB TIUXETAN

In March 2002, ibritumomab tiuxetan, an anti-CD20 murine IgG1 MAb (the parent Ab from which rituximab was derived), conjugated with Y-90 as a radiation source, was approved by the FDA to treat advanced NHL (110–112). The therapeutic regimen is delivered in two steps: step 1 includes infusion of rituximab preceding administration of In-111 ibritumomab tiuxetan; step 2, which follows step 1 by 7–9 d, consists of a second infusion of rituximab followed by Y-90 ibritumomab tiuxetan (162). The mechanisms of activity are ADCC, CDC, apoptosis, and delivery of radiation; consequently, dose-dependent hematologic toxicities are expected (110–112).

In an early phase study, 18 patients with relapsed low- or intermediate-grade NHL were treated with unlabeled anti-CD20 MAb and the Y-90 anti-CD20 MAb (ibritumomab tiuxetan) (163). Dosimetry and biodistribution analyses showed that when patients received the unlabeled Ab before ibritumomab tiuxetan, better imaging results than with the radiolabeled Ab alone, increased cumulative concentrations of the radiolabeled Ab with increased uptake at the disease sites, and decreased splenic uptake and renal excretion were observed (163). Spleen size, tumor burden, and preinfusion of unlabeled Ab were shown to affect the biodistribution of the radiolabeled Ab (163). The overall response rate was 72% in this study, with response durations ranging from 3 to >29 mo (163).

Early phase studies with ibritumomab tiuxetan administered after a short course of rituximab, in patients with low-grade, intermediate-grade, or mantle cell NHL resulted in a 67% response rate in patients with low- or intermediate-grade or mantle cell NHL and 82% in patients with low-grade NHL (111). In phase 2 studies, response rates in patients with refractory indolent lymphoma ranged from 60 to 70% (112). Additionally, similar response rates were observed in chemotherapy-sensitive and refractory patients treated with ibritumomab tiuxetan (81 and 77%, respectively) and were greater than the response rates observed in chemotherapy-refractory and chemotherapy-sensitive patients treated with rituximab (32 and 59%, respectively) (112). In a comparative study of ibritumomab tiuxetan and rituximab in patients with relapsed or refractory low-grade follicular lymphoma, the response rates were 80 and 44%, respectively (110–112,164). Notably, when the patients refractory to rituximab were treated with ibritumomab tiuxetan, an overall response rate of 46% was seen.

Generally, treatment with ibritumomab tiuxetan alone or as part of a treatment regimen including rituximab was well tolerated, with minimal nonhematologic toxicities except for infections. Hematologic toxicities included grade 4 neutropenia and thrombocytopenia. Although 4 of 18 (22%) patients treated with ibritumomab tiuxetan alone had a HAMA response months after treatment, HAMA production was not reported when ibritumomab tiuxetan was administered after a short course of rituximab (112,163).

#### TOSITUMOMAB

Tositumomab is an anti-CD20 murine IgG2a Ab conjugated with I-131 as a radiation source that has recently been approved in the United States for treatment of advanced

NHL. Similar to ibritumomab tiuxetan, the mechanisms of activity are ADCC, CDC, and apoptosis; and radiation and dose-dependent hematologic toxicities are expected (112).

In early phase studies of I-131-tositumomab in patients with chemotherapy-relapsed or refractory low-grade or transformed low-grade NHL, response rates ranged from 57 to 83%, with median duration of responses of 12–22 mo (161).

A randomized study comparing unlabeled and I-131-labeled tositumomab showed that confirmed response rates were significantly greater in patients treated with the radiolabeled Ab (55 vs 17% for the unlabeled Ab). When patients who were not responsive to the unlabeled Ab were treated with I-131-tositumomab, 89% achieved a response (165). Grade 4 neutropenia and thrombocytopenia were more common in patients treated with the labeled Ab. Increased levels of thyroid-stimulating hormone (TSH) requiring thyroid hormone supplementation were reported for a few patients who received the labeled Ab. HAMA responses were observed in 21% of the patients who received the radiolabeled Ab, 19% who received the unlabeled Ab, and in one patient who received both.

A pivotal study of I-131-tositumomab in patients with chemotherapy-refractory, low-grade, or transformed low-grade B-cell NHL reported a complete response rate of 65%, which was significantly higher than the response rate for the same patients with their previous chemotherapy regimen. The primary toxicities were neutropenia and thrombocytopenia and usually occurred 4–6 wk posttreatment. Infections and increased TSH concentrations were observed, as were transient side effects including anorexia, chills, fatigue, fever, hypotension, nausea, chills, pruritus, and vomiting. Myelodysplasia was a rare late treatment effect, and HAMA responses were observed in 8% of patients (161).

Long-term evaluations of patients with relapsed NHL treated with myeloablative doses of I-131-tositumomab and autologous stem cell transplantation showed an 86% response rate and durations of >87 mo. Reversible cardiopulmonary insufficiency was dose limiting and late toxicities were uncommon, except for increased TSH concentrations in 60% of the patients (166).

Press et al. (167) observed that patients with relapsed B-cell lymphoma treated with high-dose I-131-tositumomab, etoposide, cyclophosphamide, and subsequent stem cell transplantation had an estimated 83% survival rate and 68% progression-free survival rate at 2 yr. Patients universally experienced myelosuppression, which was ameliorated by the transplant. Other side effects included mucositis, nausea, infections, and transient abnormal liver function. More serious side effects included venoocclusive disease, deaths as the result of infections and adult respiratory distress syndrome, reversible and nonrelated increases in creatinine, increased TSH concentrations requiring thyroid hormone supplementation, and other gastrointestinal and pulmonary toxicities. Approximately 25% of the evaluated patients had a HAMA response, about one third of which were transient (167).

### **Solid Tumors**

More than 90% of all human cancers are solid tumors (168). In 1998, trastuzumab was approved for the treatment of metastatic breast cancer and to date is the only approved MAb therapeutic for solid tumors (130). Many other MAbs are in clinical development for the treatment of solid tumors. Compared with hematologic malignancies, several challenges must be overcome in the treatment of solid tumors, including expression patterns of the Ag on tumor vs normal tissue, penetration of the Ab mol-

ecules into bulky and poorly vascularized tumors; and relative responsiveness of solid tumors to Ab therapy, chemotherapeutic agents, toxins, and radiotherapy.

### *Naked Antibodies*

#### TRASTUZUMAB

Trastuzumab is a humanized MAb that was rationally designed to target and inhibit growth of breast cancer cells overexpressing human EGFR-2 (Her2) (34,83,130). In September 1998, trastuzumab was approved by the FDA as a single-agent therapy for patients with metastatic breast cancer and Her2-positive tumors who had failed previous treatments, and as a first-line therapy in combination with paclitaxel in patients with metastatic disease (130,169,170). Trastuzumab was approved in Europe in August 2000 for these same indications (169). The recommended and approved regimen of trastuzumab is an initial dose of 4 mg/kg administered intravenously over 90 min, followed by 2 mg/kg intravenously over 30 min once a week until disease progression (60;171).

Her2 receptor overexpression is dependent on amplification of the *Her2* gene (169). Patients with Her2-positive tumors generally have more aggressive disease and decreased overall survival, regardless of node status, than patients with Her2-negative tumors (130,169).

Her2 status must be accurately determined for maximum therapeutic effect of trastuzumab (169). Initially, trastuzumab was approved for the treatment of patients in whom at least 10% of the breast cancer cells overexpressed Her2, as determined by 2+ or 3+ immunohistochemistry staining (172). More recently, patient selection has been improved by using fluorescence *in situ* hybridization to determine *Her2* gene amplification (169,170).

In nonclinical pharmacology studies, trastuzumab inhibited growth of Her2-positive cells, induced immune cells to kill targeted tumor cells, and enhanced the cytotoxicity of chemotherapy agents (71). For example, when trastuzumab was administered at clinically relevant drug concentrations, synergistic activity was seen with cisplatin, thiotepa, and etoposide; additive cytotoxic effects were observed in combination with doxorubicin, paclitaxel, methotrexate, and vinblastine; and antagonistic effects were observed with 5-fluorouracil (171,173).

Nonclinical and clinical studies have shown that trastuzumab has a dose-dependent nonlinear pharmacokinetic profile that is consistent with saturation of the clearance mechanism (60). Clinical studies have shown that an initial loading dose allowed for the targeted plasma concentration to be achieved more quickly (172). These studies have also shown that, at the chosen dose, the level of shed circulating Her2 does not influence tumor response (172). Current pharmacokinetic and safety data suggest that the dose of trastuzumab can be increased and administered less frequently; therefore, studies are ongoing to evaluate a regimen of iv administration once every 3 wk, rather than weekly, and by sc rather than iv administration (60).

Clinical studies with trastuzumab administered as a single agent in patients with Her2-positive metastatic breast cancer with or without previous chemotherapy have shown durable objective tumor responses (130,170,172,174). A study of trastuzumab administered as a single agent in refractory patients resulted in a 15% objective response rate, with a median duration of response of 9.1 mo and 21% of patients free of disease progression at 6 mo (130,172). A randomized comparative study of trastuzumab in combination with standard chemotherapy or chemotherapy alone in



patients with Her2-positive metastatic breast cancer resulted in significantly prolonged median time to progression, increased overall response rates, and increased 1-yr survival times (175). Notably, quality of life was better maintained with the combination treatment when compared with chemotherapy alone (130). When trastuzumab was combined with docetaxel to treat patients with metastatic breast cancer, the overall response rate was 45% as a first-line treatment, and 54% as a second-line treatment (176).

Generally, the safety profile was acceptable for trastuzumab alone or in combination with chemotherapy (130,170,172,176–178). Cardiac dysfunction, manifested as congestive heart failure, cardiomyopathy, and/or a decrease >10% in left ventricular ejection fraction, was observed in approx 5% of patients receiving trastuzumab monotherapy and occurred more often in women who had preexisting heart conditions or high prior cumulative anthracycline exposure (130,172,179). Infusion-related reactions, including fever, chills, pain, asthenia, nausea, vomiting, and headache, were the most common side effects and were managed with simple supportive care such as acetaminophen, diphenhydramine, and, if necessary, meperidine (171,172,179). Between 1998 and 2001, 74 reports of serious, treatable infusion-related events were reported worldwide among approx 25,000 treated patients (96). It is recommended that physicians monitor patients for the main side effects: cardiac dysfunction and serious infusion-related reactions (96).

Ongoing studies in the United States and Europe include trastuzumab in the adjuvant setting in patients with primary breast cancer (180–183). In addition, other therapeutic strategies for combination studies with trastuzumab include growth factor inhibitors, receptor inhibitors, and direct inhibition of other intracellular signaling pathway components (184).

#### MAbs TARGETING EGFR

EGFR is a 170-kDa glycoprotein with an extracellular ligand-binding domain (LBD), a transmembrane lipophilic segment, and an intracellular tyrosine kinase domain (185). On ligand binding, members of the EGFR family dimerize with subsequent protein tyrosine kinase activation and tyrosine autophosphorylation, stimulating downstream effectors of cell growth (186,187). EGFR signaling activates a pathway that promotes tumor proliferation, migration, stromal invasion, neovascularization, and resistance to apoptosis (188) (Fig. 5). Overexpression of EGFR has been shown in multiple tumors, including cancers of the breast, ovary, colon, prostate, kidney, head and neck, and lung (189–192). Additionally, overexpression of EGFR has been associated with resistance to hormonal therapy, cytotoxic agents, and radiotherapy (193). Furthermore, EGFR ligands, including EGF and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) are upregulated in numerous malignancies (194).

One therapeutic strategy is to block EGFR-driven autocrine growth pathway, thereby impeding development and progression of the targeted cancers (186). Another strategy is to use bispecific Abs to induce association of immunologic effector cells to the receptor (184). To date, the most promising strategies to inhibit EGFR signaling have been small-molecule intracellular inhibition of tyrosine phosphorylation or MAbs directed against the extracellular LBD of the receptor (186).

#### CETUXIMAB

Cetuximab is a chimeric MAb of the IgG1 isotype with a binding affinity ( $K_D$   $2 \times 10^{-10}$  M) for EGFR that is greater than that of EGF or TGF- $\alpha$  (184,195). Cetuximab

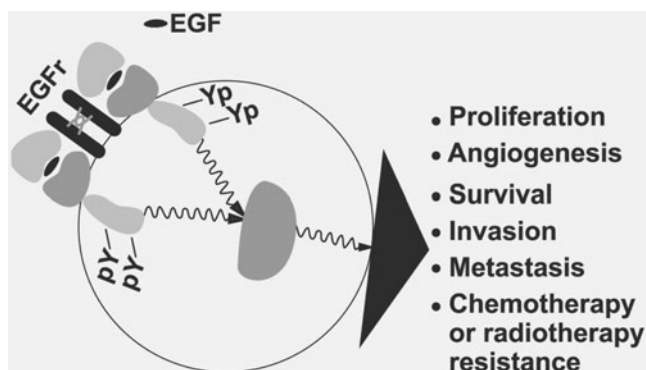


Fig. 5. EGF and TGF- $\alpha$  bind to the EGFR, resulting in dimerization and receptor tyrosine phosphorylation with activation of downstream growth signals and promoting cell proliferation and metastasis.

promotes receptor internalization, reducing the number of receptors available for binding; inhibits cell-cycle progression, causing cell-cycle arrest before DNA synthesis; and inhibits EGF-induced secretion of angiogenic factors in a dose-dependent manner (195–197). In addition, cetuximab inhibits the expression and activity of several MMPs that play a role in tumor cell adhesion, and it has been correlated with a significant reduction in tumor cell invasion *in vitro* and inhibition of tumor growth and metastasis *in vivo* (195). Cetuximab administered as a single agent induces apoptosis in some cell lines, whereas other cell lines require cetuximab in combination with chemotherapeutic agents (195,198).

Cetuximab has a dose-dependent, nonlinear pharmacokinetic profile (195,198). Its profile is not altered when administered with chemotherapeutic agents such as cisplatin (193). The dose being evaluated in clinical trials is an initial loading dose of 400 mg/m<sup>2</sup> followed by weekly maintenance infusions of 200 or 250 mg/m<sup>2</sup> (195).

The primary indications in which cetuximab has been studied are colon cancer and head-and-neck cancer, with cetuximab administered as a single agent or in combination with chemotherapy or radiotherapy (184,199). When cetuximab was administered as a single agent in patients with advanced irinotecan-refractory colorectal cancer, a response rate of 11% was reported (200). Data from an earlier study in patients with advanced colorectal cancer suggested that 22.5% of patients achieved a partial response, and an additional 7% achieved stable disease for >12 wk when cetuximab was combined with irinotecan (199,201). A randomized clinical study of cetuximab in combination with cisplatin in patients with head-and-neck cancer resulted in a 2% complete response rate, an 11% partial response rate, a 73% stable disease rate, a median disease-free duration of 6.7 mo, and an overall survival duration of 7.2 mo. These results were not statistically different from those observed with cisplatin alone (202). Phase 2 studies of cetuximab administered as a single agent or in combination with chemotherapy have been done in patients with renal cell cancer, pancreatic cancer, and non-small cell lung cancer (NSCLC) (203–205). Late-stage studies are currently ongoing to evaluate the activity of cetuximab as a single agent and as part of a combination therapy with chemotherapy or radiotherapy in patients with colorectal cancer or head-and-neck cancer, respectively (201).

Up to 80% of patients treated with the recommended regimen of cetuximab have experienced an acneform rash (193,195,196). Anaphylactic reactions have been described in 1–2% of patients, primarily with the first infusion (206). Additionally, grade 3 and 4 allergic reactions were reported in approx 4% of patients (195), and 5–6% of the patients developed a HACA response.

### ABX-EGF

ABX-EGF is a fully human IgG2 MAb specific to human EGFR that was developed using XenoMouse® technology, a transgenic mouse technology allowing for the generation of fully human Abs (207). Fully human MAbs generated by XenoMouse® strains are anticipated to be nonimmunogenic, and therefore allow repeated administration without HAHA responses (207). An IgG2 isotype was chosen to avoid potential safety issues resulting from recruitment of effector function (ADCC) to normal tissue that overexpresses EGFR. ABX-EGF is being studied in a comprehensive phase 2 program as monotherapy and in combination with standard chemotherapy.

ABX-EGF has a high affinity for EGFR ( $K_D$   $5 \times 10^{-11}$  M), blocks binding of EGF and TGF- $\alpha$ , and inhibits tyrosine phosphorylation and tumor cell proliferation in a dose-dependent fashion (207). In vitro studies showed that ABX-EGF inhibited tumor cell growth (208). When ABX-EGF was evaluated in xenograft tumor models of cell lines overexpressing EGFR, tumor formation was prevented, tumor growth was inhibited, and established tumors were eradicated (207). Furthermore, additive or synergistic antitumor effects were observed when ABX-EGF was administered in combination with chemotherapeutic agents (209).

In a phase 1 study, ABX-EGF was generally well tolerated, with low pharmacokinetic variability. Clearance of ABX-EGF was dose dependent due to the progressive saturation of EGFR (102). The incidence of acneform skin rashes was dose dependent, and all patients who received at least one dose of ABX-EGF, 2.5 mg/kg, developed an acneform skin rash.

Preliminary data from this phase 1 study and a phase 2 study in patients with advanced renal cell cancer suggest activity against the malignancy with low doses of ABX-EGF (108,109). Of the 46 patients included in the phase 1 study, partial responses or stable disease/minor responses were observed in 6 patients ([108,109]; unpublished data). In the phase 2 study, objective responses were noted in 3 of 88 patients eligible for response evaluation, and 46 of 88 achieved a minor response or stable disease (109). Similar to other studies, the most common side effect was low-grade transient acneform skin rash (109). No allergic reactions, infusion-related reactions, or serious adverse events have occurred to date, and no HAHA responses have been detected ([102,108]; unpublished data).

### OTHER EGFR-TARGETING ANTIBODIES

Other MAbs targeting EGFR that are currently in clinical development include two humanized Abs, hR3 and EMD 72000, both of which are of the IgG1 isotype (210,211). Preliminary data with hR3 in combination with radiotherapy in patients with locally advanced squamous cell head-and-neck cancer indicate that the Abs may enhance the response to radiotherapy in this patient population (210). A phase 1 study with EMD 72000 in patients with solid tumors expressing EGFR resulted in a 28% partial response rate, 22% stable disease rate, and dose-limiting toxicity of headache and fever, which indicated that the maximum tolerated dose was 1600 mg/wk (211). Interestingly, the

typical dose-related skin rash reported with small molecules targeting EGFR, ABX-EGF, and cetuximab was not observed with hR3 (210).

#### ANTIBODIES TARGETING EGFRvIII

The most common mutant of EGFR in human cancers is EGFRvIII, a truncated EGFR that is not found on normal cells and is not capable of ligand binding due to missing domains in the extracellular domain (193,196). EGFRvIII lacks 267 amino acids normally found in the extracellular domain of wild-type EGFR (212–215). EGFRvIII does not depend on ligand interaction for cell stimulation, and amplification results in expression on tumor cells (193,196). EGFRvIII is commonly found in glioblastoma multi-forme and has been reported in other carcinomas, including breast, ovarian, prostate, and lung, but not on normal tissues, and is therefore a tumor-specific target (212–215).

Anti-EGFRvIII MAbs are being investigated in experimental model systems (212,213). Y10 is a murine MAb against mutated EGFRvIII that inhibits cellular proliferation, induces cell-mediated cytotoxicity in vitro, and increases survival in in vivo models (193). In its unconjugated form, Y10 is effective against EGFRvIII, as evidenced by increased survival rates and >25% cure rate in tumor-bearing mice (212,213,215).

Because EGFRvIII is a tumor-specific target and Abs against it are internalized rapidly to intracellular vesicles, conjugation with immunotoxins or radioisotopes is feasible and promising (212,213). Conjugation of the anti-EGFRvIII MAb, scFv MRI-1, with *Pseudomonas* exotoxin showed promising results (212,213,215). Specifically, median survival times were increased dramatically, from 430 to 657%, in tumor-bearing rats, with long-term survival dependent on the toxin dose. Furthermore, this Ab, when radiolabeled, showed enhanced tumor retention, rapid clearance from the blood, and high tumor-to-normal tissue ratios (212,213).

#### ANTIBODIES TO VEGF

Angiogenesis is a critical determinant for the growth, invasion, and metastasis of many cancers (216). A strategy designed to attack the endothelial lining of the vasculature in solid tumors, rather than the tumor itself, depends on identification of markers in the tumor vasculature, but not in normal tissue vasculature (168). VEGF and VEGFR are some of the most specific tumor vasculature markers (168).

VEGF-A is a highly specific, selective mitogen for vascular endothelial cells in vitro, and is produced in vivo in many tissues (168,216–218). It is required for vasculogenesis, is a primary stimulant of angiogenesis, enhances vascular permeability, and protects against apoptosis in newly formed blood vessels (168,216–218). The activity of VEGF is induced by oncogenic mutations and the hypoxic environment of tumors and is regulated by two receptors, VEGFR1 and VEGFR2, which are expressed only in the vascular endothelium (168,218). Upregulation of the receptors is dependent on increased ligand concentrations, hypoxic conditions, and VEGF (168,217).

In preclinical studies, anti-VEGF Ab therapy enhanced the antitumor activity of radiotherapy and reversed VEGF-induced vascular permeability; this resulted in decreased interstitial fluid pressure and increased partial oxygen pressure (217).

Clinical development is ongoing for several anti-VEGF Abs and other inhibitors of VEGFR signaling (217). Bevacizumab is a humanized Ab against VEGF being investigated in late-phase studies in breast cancer, colorectal cancer, renal cell cancer, and

lung cancer (217,219). Results from phase 1 and 2 studies indicated that this Ab was generally well tolerated at various doses; however, incidences of fever, chills, headache, hypertension, infection, rash, and nosebleeds were greater in patients treated with the Ab than in those treated with chemotherapy alone (218). Although the Ab has been generally safe and well tolerated in various types of cancer, fatal bleeding complications have been observed in patients with NSCLC of the squamous-cell type treated with bevacizumab in combination with chemotherapy (219). Pharmacokinetic analyses showed that the half-life for bevacizumab is similar to that of other humanized Abs directed to soluble targets, allowing for an every-other-week administration regimen (219).

A randomized phase 2 study examining bevacizumab in patients with advanced renal cell cancer who failed prior biotherapy showed a statistically significant prolongation in time to disease progression compared with placebo (220). However, Miller et al. (221) recently reported results of a randomized phase 3 study of bevacizumab in combination with capecitabine in 462 patients with advanced breast cancer that showed no Ab effect on time to progression, or 12-mo survival, compared with capecitabine alone. One secondary end point, overall response rate, reached statistical significance (221). Further phase 3 studies are evaluating bevacizumab in combination with chemotherapy in colorectal, breast, and NSCLC. Very recently (ASCO 2003) a very significant improvement in response rate, time to progression, and survival has been reported for the combination of bevacizumab with IFL chemotherapy compared to chemotherapy alone in first line treatment of colorectal cancer.

Preclinical studies of MAb against VEGFR1 and VEGFR2 showed activity against various tumor types, including a neuroblastoma model. When MAb against the receptors were combined with vinblastine, tumors underwent full and sustained remissions without evidence of increased toxicity or drug resistance (219).

### *Immunotoxins*

Several immunotoxins are being evaluated in the clinic for the treatment of solid tumors, although none have yet reached late-stage clinical development. Examples include the following: BB-10901 is a humanized murine MAb bound to maytansinoid cytotoxin DM1 that is being evaluated in a phase 1 study in patients with relapsed or refractory small cell lung cancer (SCLC) and other neuroendocrine tumors (222). SB 408075 is a tumor-activated immunoconjugate targeting the C242 CanAg antigen with a maytansinoid payload that is being evaluated in patients with colorectal cancer (223). SGN-15 is another Ab drug conjugate that targets doxorubicin to Lewis-Y-expressing tissue that is currently in phase 2 development for several types of solid tumors (224).

### *Radioimmunotoxins*

To date, early phase dose-escalation studies of radioimmunotherapies have not shown sufficient antitumor activity of radioimmunotherapy to progress into late clinical development for large-volume malignancies, including glial tumors, leptomeningeal cancer, ovarian carcinoma, colorectal cancer, breast cancer, prostate cancer, bladder cancer, renal cell carcinoma, medullary thyroid cancer, hepatocellular carcinoma, and SCLC (225). Compared with lymphoid malignancies, solid tumors are less radiosensitive and thus require higher doses of radiation administered in single-dose or repeat-dose regimens to be effective, which can result in unacceptable toxicities (159,225).

Potential activity of radioimmunotherapies has been observed in studies of small-volume or micrometastatic disease, such as colorectal or ovarian cancers, or with locoregional administration such as neuroblastoma or leptomeningeal cancer (225). Other strategies include combination therapies, fractionated dose schedules, and pretargeting methods using bispecific Abs and radiolabeled haptens (225).

## Conclusions

The past 20 yr have brought significant advances in the ability to develop Abs that satisfy very specific design goals. One can now select Abs or Ab fragments with defined affinities, isotypes, and pharmacokinetic profiles. The understanding of and ability to deliver a payload, drug, toxin, or radiation has greatly increased and has led to recent approval of such conjugated Abs. Future development of Ab therapeutics will be facilitated by those insights gained to date. Identification and validation of new targets and improved targeting of validated targets provide a vast area for the development of optimized Ab therapeutics in the future.

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# 18

## Emerging Molecular Therapies

### *Drugs Interfering with Signal Transduction Pathways*

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**Richard Baird and Paul Workman**

#### **Introduction**

The development of innovative cancer drugs has generated much excitement. We stand poised to take advantage of our newfound understanding of the molecular basis of cancer to develop innovative treatments that are both more effective and less toxic than “traditional” cytotoxic chemotherapy (1).

The prospect of more-selective, target-based therapies is made possible through a detailed understanding of the molecular differences in structure and function between cancer and normal cells (2–5). This understanding has been achieved primarily in the last quarter of the twentieth century by painstaking, hypothesis-driven molecular biology and genetic research (6,7) but has progressed rapidly with the sequencing of the human genome (8,9). The sequencing should be finalized shortly, 50 yr after Watson and Crick elucidated the structure of DNA and the molecular mechanism DNA replication and heredity (10,11). The impact of their discovery has been reprised in a recent series of articles (12).

The impact of the new wealth of genomic information will be enormous and includes an emphasis on integrating traditional hypothesis-driven research with genomics, proteomics, and other “-omic” technologies. Discovering critical nodes in tumor cell rewiring is clearly one of the roles for “omics” research—the study of biologic systems on a global, massively parallel basis (3–5,13–15).

In this chapter, we describe the rationale for targeting signal transduction pathways, particularly in relation to our emerging understanding of the cancer genome. We describe progress with signal transduction inhibitors that are approved or in preclinical and clinical development (Table 1), including the challenges of clinical trials with such agents.

#### **Rationale for Targeting Signal Transduction Pathways**

Signal transduction describes the processes involved in the communication between the cell and its environment, and in the regulation of cell fate (16). These pathways are commonly hijacked by the genomic abnormalities that drive malignant progression (17,18). Pathways whose importance have been clearly established in cancer are illustrated in Fig. 1 (7).

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**Table 1**  
**Examples of Current Genomic Targets and Drugs in Preclinical Development**

Target	Drug	Stage in development	References
Bcr/Abl kinase Mutated c-KIT RTK PDGF RTK	STI571 (Imatinib )	FDA approval: May 2001 for CML, February 2002 for GIST	(24,49)
PML-RAR	ATRA (Vesanoïd)	FDA approval: November 1995 for APL	(25,186)
EGF (ErbB 1) RTK	MAbs		(77)
	IMC-C225 (Cetuximab)	Phase 3	
	ABX-EGF	Phase 2	
	MDX447 (EMD 82633)	Phase 1	
	Small-molecule inhibitors		
	ZD1839 (Gefitinib)	FDA approval: May 2003 for NSCLC resistant to platinum and docetaxel	
	OSI-774	Phase 3	
	CI-1033 (PD183805)	Phase 2	
	EKB-569	Phase 1	
	PK1166	Phase 1	
HER2 (ErbB2) RTK	MAbs		(77)
	Trastuzumab	FDA approval: September 1998 for HER2 positive breast cancer	
	2C4 (Pertuzumab)	Phase 1	(195)
	Small-molecule inhibitors		
	GW2016	Phase 1	
	AG-1478	Preclinical	
	R115777	Phase 3	(187)
	SCH66336	Phase 2	
	L-778123	Phase 1	
Farnesyltransferase	BMS-214662	Phase 1	
	L-744832	Phase 1	
Ras-Raf-MEK-MAPK pathway RAF1 tyrosine kinase MEK kinase	BAY 43-9006	Phase 2	(188)
	CI-1040 (PD184352)	Phase 2	(152)
	PD 98059	Preclinical	



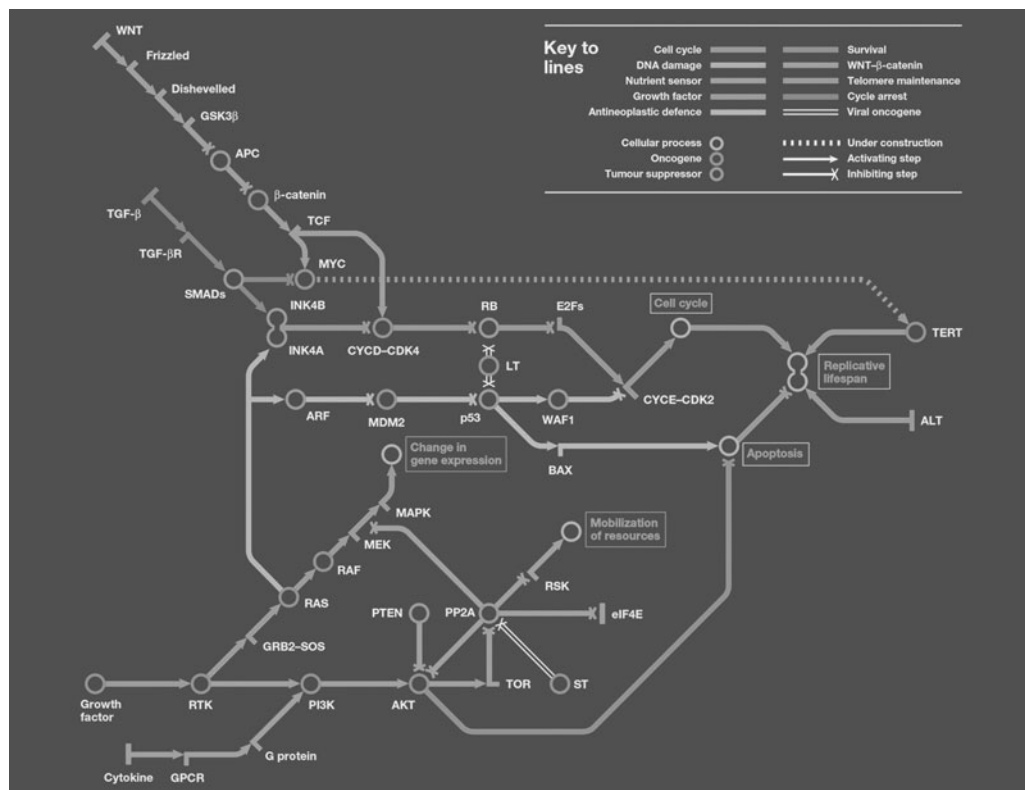


Fig. 1. Subway map of cancer pathways (193). Reprinted by permission from *Nature Reviews Cancer*, copyright (2002) Macmillan Magazines Ltd. Available online at: [http://www.nature.com/nrc/journal/v2/n5/weinberg\\_poster/](http://www.nature.com/nrc/journal/v2/n5/weinberg_poster/), with links to seminal papers and NCBI LocusLink entries for each gene product. (Color illustration in insert following page 362.)

The rationale for developing signal transduction inhibitors as anticancer agents is clear: drugs that are targeted to crucial molecular abnormalities and biochemical pathways exploited by cancer cells should be more effective and less toxic to normal tissues than the broadly antiproliferative cytotoxic drugs that dominate current therapy (2,19).

Thus, new molecular targets selected for drug development should be those that are important for the initiation and progression of cancer, e.g., those targets causing activation of proliferative signal transduction pathways; activation of antiapoptotic, cell-survival pathways; and pathways involved in the initiation of angiogenesis, invasion, and metastasis (7,17,20–22). This promising approach is in its infancy but is encouraged by the clinical progress of a number of drugs in the “first wave” of molecular therapeutics, such as imatinib mesylate, all-*trans* retinoic acid (ATRA), and trastuzumab.

Imatinib mesylate (also known as STI571) targets the BCR-ABL fusion protein in chronic myelogenous leukemia (CML) and the mutated KIT receptor in gastrointestinal stromal tumors (GIST) (23). ATRA targets the PML-retinoic acid receptor  $\alpha$  (PML-RAR $\alpha$ ) translocation product in acute promyelocytic leukemia (APL) (24).

Trastuzumab targets the ErbB2 (HER2-neu) receptor commonly found in breast cancer. These agents have achieved high clinical response rates when used as single agents in patients with the targeted molecular defect, and are considerably less toxic than conventional combination cytotoxic chemotherapy. They clearly show the clinical potential of new drugs targeted to cancer-specific, deregulated, signal-transduction pathways.

## Strategies for Hitting Signal Transduction Targets

The main focus of this chapter is the development of small-molecule drugs (generally defined as having a molecular weight <500 kDa). Selected antibodies (Abs) are considered where these have mechanistic relevance. In addition, several alternative strategies show considerable promise, including the use of antisense oligonucleotides (25), protein therapies (26), gene therapy (27), cancer vaccines (28), and RNA interference (RNAi) (29). Readers are directed to the recent reviews indicated.

Compared with other strategies, small-molecule drugs have several advantages and disadvantages. They have attractive pharmacokinetic properties, particularly for oral administration and tumor penetration, and are relatively easy to produce. On the other hand, it is technically very difficult to design small molecules to successfully disrupt large-domain protein–protein interactions (e.g., SH2 domains), or to interfere with transcription factor–DNA complexes. Nonetheless, small-molecule drugs have a proven track record of successfully inhibiting enzymes, including classic targets such as dihydrofolate reductase and more novel kinase targets (imatinib mesylate, gefitinib, and others).

## Contemporary Drug Development

Historically, drug development has been an expensive, slow, and risky business. Taking into account the cost of failed drugs, the estimated cost of drug development is US\$500 to US\$800 million for each successful agent. Even omitting the cost of failure, it requires nearly US\$100 million to develop a single drug.

The entire process has, in the past, taken an extremely long time; the average development period is approx 15 yr to progress a drug from initial discovery to regulatory approval, and it can take much longer. A stark example is provided by the development of paclitaxel, which received regulatory approval in 1992, 29 yr after the crude extract from the bark of the Pacific yew tree was discovered to have antitumor activity. Another example is the delay between the discovery of the *RAS* oncogene in 1982 and the start of clinical trials with a *RAS* antisense oligonucleotide and farnesyltransferase inhibitors (FTIs) in 1999.

The riskiness of drug development is apparent from the fact that only 1 in 10 drugs entering clinical trials will attain regulatory approval. The failure rate in preclinical development is considerably higher, with perhaps only 1 in 100 drugs making it into the clinic. Frequent reasons for failure include poor pharmacokinetics, toxicity, and lack of efficacy.

How, then, do researchers go about developing signal transduction inhibitors for use as anticancer agents? How do researchers create more effective, less-toxic drugs, quicker (in 5–7 yr or less), more efficiently, and less expensively? It seems an impossible task. The key to recent successes has been to focus efforts on important molecular targets, and to take advantage of a range of new technologies to accelerate the drug discovery process (2,3,30,31).

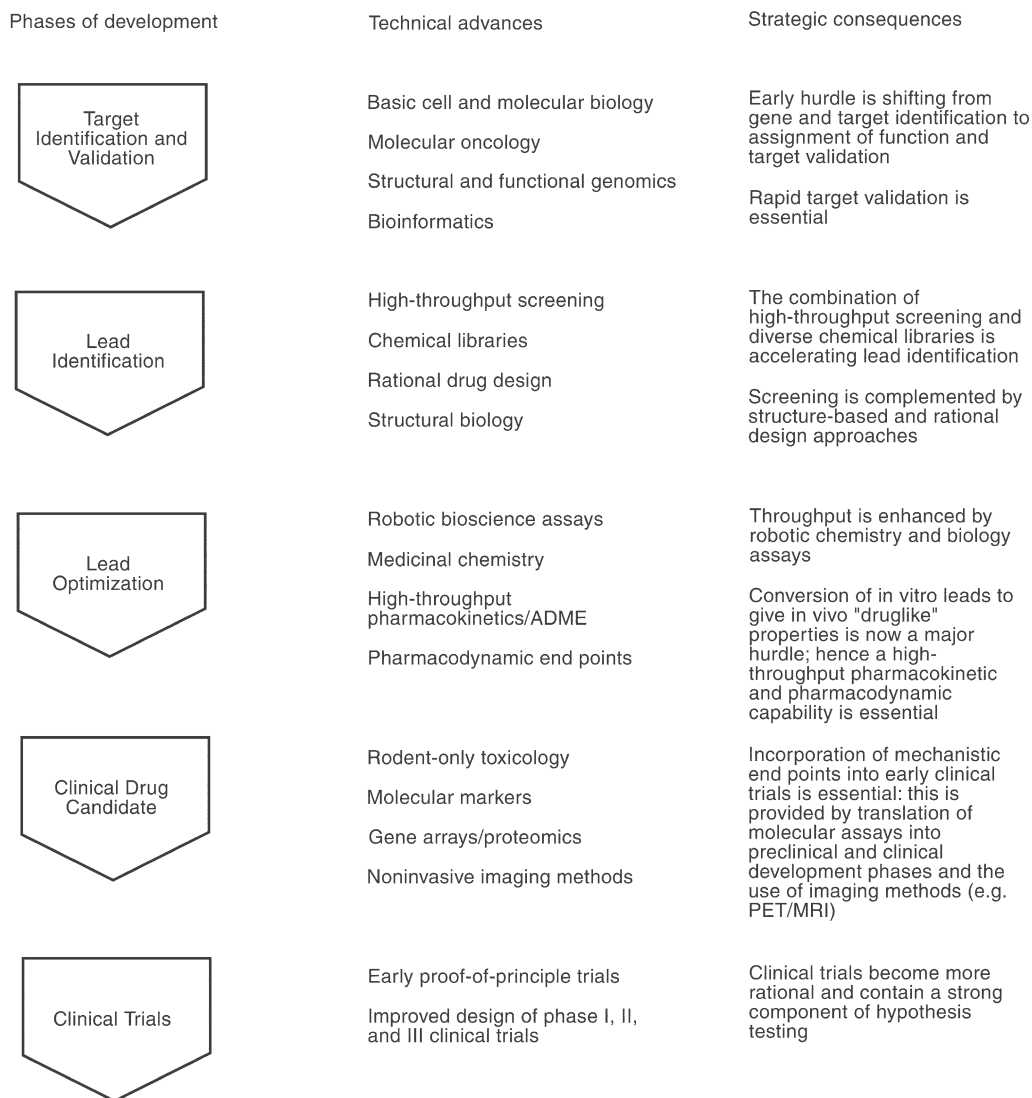
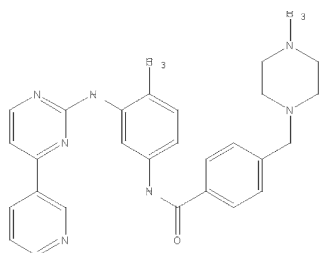


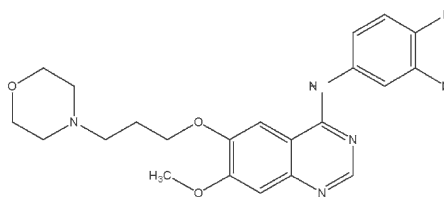
Fig. 2. Contemporary drug development. ADME, absorption, distribution, metabolism, and excretion. Note: Rodent-only toxicology has been advocated based on the successful experience by Cancer Research UK and the European Organisation for the Research and Treatment of Cancer (32). (Modified from ref. 31.)

## New Technologies Enhancing the Efficiency of Drug Discovery and Development

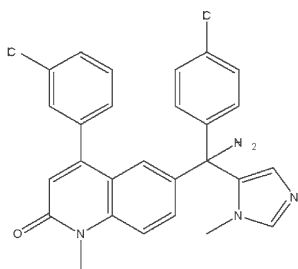
Figure 2 illustrates the phases of contemporary drug development with the key new technologies that facilitate the process. The completion of the Human Genome Project, together with the Cancer Genome Project and associated activities, will accelerate the stream of new targets flowing into cancer drug discovery. This systematic, high-throughput sequencing of the genomes of cancer cell lines and patient tumors should uncover the remaining cancer genes and will be a rich resource to mine for drug development targets



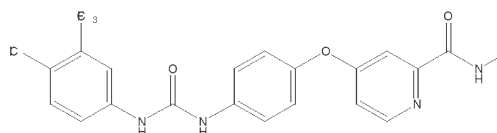
STI571 (Imatinib)



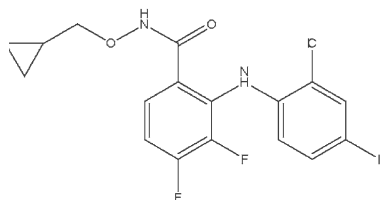
ZD1839 (Gefitinib)



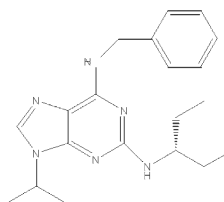
R115777



BAY 43-9006



CI-1040 (PD184352)



CYC202 (R-roscovitine)

Fig. 3. Selection of new drug structures.

over the next 5–10 yr. Table 1 provides examples of the range of current genomic targets for drugs in development, and Fig. 3 illustrates a selection of new drug structures.

One early success for the Cancer Genome Project was the identification of activating mutations in the *BRAF* gene found to be present in 70% of melanomas, 10% of colorectal cancers, and a number of other tumors (33). This finding provides strong evidence to support the development of inhibitors of the *BRAF* kinase.

How will researchers know which genes in the rich torrent of information flowing from cancer genomes are the most important on which to focus their efforts? It is possible that many mutated or deregulated cancer genes will be downstream consequences of genomic instability and occur as relatively late changes in the stepwise process of multistep carcinogenesis. Neutralizing the effects of such genes may well not turn around

**Table 2**  
**Criteria for Validation and Selection of New Drug Targets**

- 
- Frequency of genetic or epigenetic deregulation of the target or pathway in human cancer
  - Demonstration in a model system that the target contributes to the malignant phenotype
  - Evidence of reversal of the malignant phenotype (e.g., by gene knockout, dominant negative, antisense, RNAi, antibodies, peptides, or drug leads)
  - Practical feasibility, tractability, or drug ability of the target (e.g., enzymes are commonly more tractable than most protein–protein interactions)
  - Availability of a robust, efficient, and informative biologic test cascade to support the drug discovery program
  - Ability to build and run a cost-effective high-throughput screen
  - Availability of a structure-based design approach
  - Potential for the use of pharmacodynamic end points and other biomarkers for diagnosis and outcome prediction
- 

Modified from ref. 3.

the cancer juggernaut, whereas targeting those genes driving early carcinogenesis could be more effective, especially for early intervention and chemoprevention. On the other hand, these approaches require successful early diagnosis. Many factors influence the choice of which molecular target to work on. Table 2 outlines the criteria for selecting and validating new targets for contemporary drug development.

Gene expression microarrays have helped speed up the process of target identification and validation, and the ability to profile the messenger RNA transcribed from thousands of genes simultaneously enables genomic and mechanistic questions to be addressed at all stages of drug development (34). Proteomic technology is less well advanced for routine use at present, but clearly the ability to measure the expression of thousands of genes at the functional protein level will be extremely valuable (35,36). The next important step is to understand the complexity of interconnecting, dynamic signal transduction pathways in similar detail, and to produce mathematical models to describe these and predict the consequences of intervention (37). The application of high-throughput “omics” technologies provides a global picture of the genes and pathways that are deregulated in oncogenesis. Their integration with a traditional hypothesis-driven approach has particularly powerful potential (4,6,14–16,38).

Automated high-throughput screening is the major source of novel drug leads and is used to rapidly identify inhibitors of a specific target. High-throughput screening is achieved by screening large, diverse, chemical libraries (20,000 to >100,000 compounds) against either recombinant proteins or cells that have been engineered (e.g., to have a reporter-gene readout of a particular signal transduction pathway). In addition to “real,” robotic high-throughput screening, virtual “in silico” screening methods (39), rapid fragment screening by nuclear magnetic resonance, and high-throughput crystallography (40) can all be used together synergistically to increase the likelihood and efficiency of finding hits. The ability to generate large numbers of molecules for further high-throughput screening has been enabled by modern chemistry techniques, including solid-phase, combinatorial, and parallel synthesis (41).



Initial hits often have low potency and selectivity for the target and will require chemical modification to generate improved lead compounds (as in the initial development of imatinib mesylate). To this end, the “hit to lead” process involves close interaction of bioscientists with medicinal chemists who refine the structure of the hit compound in multiple rounds of making and testing, guided by biologic feedback.

It is important to note at this stage that a great proportion of compounds that fail in the drug development pipeline do so because they are unable to make the transition from biochemical or cell-based assays to successful use in preclinical or clinical studies. This failure is usually because, despite having good potency and selectivity against the molecular target and exhibiting promising properties in cells, they have poor pharmacokinetic properties, and hence will never make it to the target in sufficient concentrations in vivo. One key to successful drug development is to focus at a fairly early stage on the pharmacokinetics of new compounds and their potency and selectivity on the target. The high-throughput screening and synthetic techniques that we have discussed are supported by high-throughput cassette- or cocktail-dosing techniques, in which the pharmacokinetics of a mixture of compounds, administered to animals in low doses, can be determined using high-performance liquid chromatography coupled to mass spectrometry (4).

The use of such new technologies accelerates the development of potent, specific, and “drug like” compounds designed to modulate specific molecular targets, including various components of cancer signal transduction pathways.

## Clinical Trial Design

Once a potent, selective, and “druglike” signal transduction inhibitor is designed, how are clinical trials initiated? The drug has shown anticancer activity in vitro and antitumor activity in animal models (e.g., human tumor xenografts), but how do researchers know what dose and schedule to use, and how will they know if it is inhibiting the target in patients?

### **Traditional Clinical Drug Development for Cytotoxic Agents**

It has become clear that the traditional model of early clinical trials (42) (summarized in Table 3) is not particularly well suited to the assessment of molecularly targeted agents, for several reasons.

Traditional phase 1 trials of cytotoxic drugs are designed to find the maximum tolerated dose (MTD), to identify the range of toxicities seen, including the dose-limiting toxicity (DLT), and to establish the recommended dose (RD) for further clinical trials. Any hints of tumor response are documented but investigated further in subsequent trials. Pharmacokinetics behavior is likely to be characterized.

Phase 2 trials are done in specific tumor types, treating patients with the RD identified in phase 1 studies. The main end point is tumor response, which is based on tumor size. Guidelines developed by the World Health Organization (WHO) in the late 1970s define tumor response in four categories. Complete response requires disappearance of the tumor. Partial response is defined as a reduction in size of the tumor  $\geq 50\%$ . Progressive disease is defined as an increase in tumor size of  $\geq 25\%$ . Stable disease means neither partial response nor progression disease criteria have been met, indicating tumor stasis but no significant change in tumor size.

The WHO guidelines were reviewed in the light of three decades of clinical use and have been updated in the Response Evaluation Criteria in Solid Tumours (RECIST) guidelines (43). Important differences in response criteria are outlined in Table 4.

**Table 3**  
**Traditional Clinical Drug Development**

	Phase 1	Phase 2	Phase 3
Objective	What is the RD to take forward to phase 2/3? Range of toxicities and MTD Pharmacokinetics	Is there antitumor activity in selected tumor types?	Is the new treatment better than current standard therapy?
Disease	All tumor types	Tumor type specific	Tumor type specific
Dose	Escalated	RD	RD
End point	Toxicity	Tumor response	Survival Symptoms + quality of Life
Design	Dose escalation in small cohorts (3–6 patients)	Two-stage (early stopping rule)	Randomized, with or without blinding

Modified from ref. 194.  
MTD, maximum tolerated dose; RD, recommended dose.

**Table 4**  
**Definition of Best Response According to WHO and RECIST Criteria**

Best response	WHO change in sum of products	RECIST change in sums of longest diameters
CR	Disappearance	Disappearance
PR	50% decrease	30% decrease
SD	Neither PR nor PD criteria met	Neither PR nor PD criteria met
PD	25% increase	20% increase

CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

Phase 3 trials are designed to answer the question: Is this new therapy better than the current standard therapy? These definitive studies are usually randomized, controlled trials involving hundreds of patients, often recruited from many different institutions, and are expensive and time-consuming to complete. The end points in phase 3 studies are survival, patient symptoms, and quality of life. As such, they represent “true” end points (as opposed to surrogate end points, such as tumor shrinkage).

### ***Clinical Trial Design for Molecular Therapeutics***

Several important differences in the properties of molecularly targeted agents demand a new approach to clinical trials (Table 5). First is the question of dose. Drugs acting on highly specific molecular targets that are differentially expressed or activated in cancer cells may result in relatively low tissue toxicity, and where toxicity is seen, this may involve nonproliferating tissues. Such drugs may reach an optimum biologic dose that is significantly below the MTD, and this can be assessed by measuring pharmacodynamic markers of biochemical or biologic activity in tissue samples from patients.

Second is the question of schedule. In many current front-line treatment regimes, cytotoxics are administered at their MTD in a pulsed fashion, each pulse having the maximum antitumor effect, but also causing considerable toxicity. Breaks between cycles allow for recovery of normal tissues, particularly bone marrow. By contrast, molecular therapeutics are likely to be cytostatic, rather than cytotoxic, and may be more effective if administered continuously (preferably by the oral route), rather than in intermittent iv pulses.

Third is the question of patient selection. Who should receive the drug? Traditional phase 1 studies enrolled patients with a wide range of tumor types, while a phase 2 study examined tumor response in patients with a particular histologic type (e.g., non-small cell lung cancer [NSCLC]). With molecularly targeted agents, it may be necessary in many cases to identify a subset of patients who are most likely to respond, e.g., based on expression or mutation of the target in a deregulated signal transduction pathway. The most prominent examples of this in current practice are (1) the use of trastuzumab in patients with breast cancers that overexpress the ErbB2 (HER2-neu) receptor (44) and (2) the use of imatinib mesylate in the treatment of patients with leukemia, targeting the BCR-ABL translocation, as well as patients with GISTs with activating KIT mutations (24). Insufficient data may be available to restrict entry in phase 2 or 3 studies to patients with a high level of expression or mutation of the target, but it should be essential that studies collect data on the target status to correlate this with outcome. Development of an understanding of the relationship between response and the status of the molecular target, or indeed other molecular biomarkers, may lead to a closer definition of the appropriate patient populations for later trials.

Fourth is the question of tumor response. Phase 2 studies have, in the past, used tumor shrinkage as a surrogate outcome measure for clinical benefit, but given the predominantly cytostatic effects of most molecular therapeutics, significant tumor shrinkage is unlikely. If, however, these agents can offer patients an increase in time to progression or stabilization of their disease, these criteria can be used as end points in phase 2 and 3 trials.

Finally, more flexible and creative trial designs, those that are tailored to the specific properties of the drug being studied, such as combined phase 1–2 trials and randomized discontinuation studies, may be needed (45).

**Table 5**  
**Clinical Drug Development of Target-Based Therapy**

	Phase 1	Phase 2	Phase 3
Objective	Optimum biological dose Pharmacokinetics Pharmacokinetic-pharmacodynamic relationship	Is there antitumour activity in selected tumor types?	Is the new treatment better than current standard therapy?
Disease	Range of toxicities		
Dose	Target-bearing tumors Escalated	Target-bearing tumors Optimum biologic dose	Target-bearing tumors Optimum biological dose
End point	Inhibition of target	Time to progression	Survival
Design	Guided dose escalation	Controlled	Randomized ( $\pm$ blinded)

Modified from ref. 194.

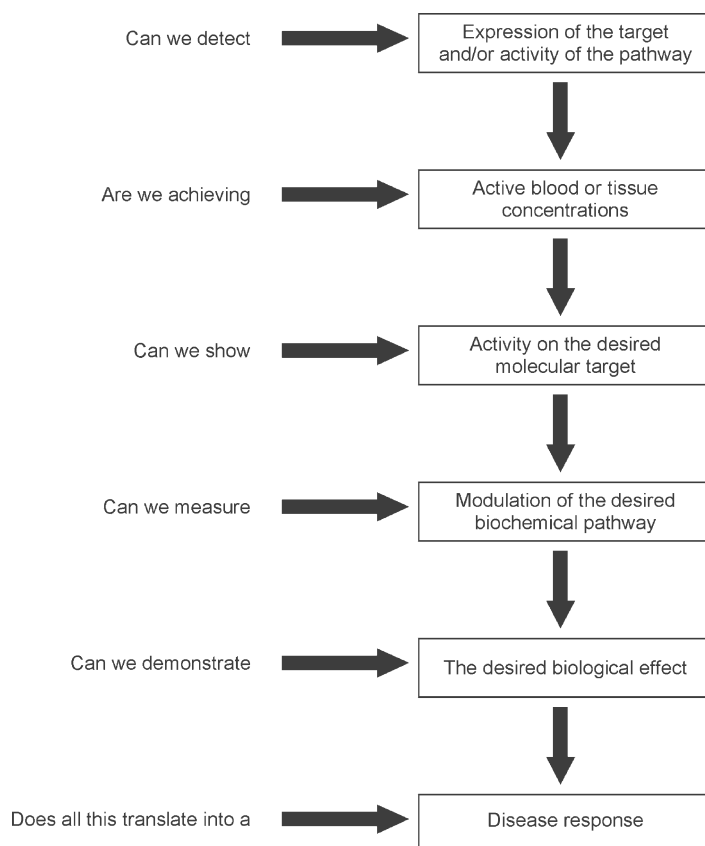


Fig. 4. Pharmacologic audit trail: the importance of pharmacokinetic and pharmacodynamic end points. (Modified from ref. 47.)

### ***Importance of Pharmacokinetic and Pharmacodynamic End Points***

To properly evaluate molecularly targeted drugs, it is necessary to define both what the body does to the drug (pharmacokinetics) and what the drug does to the body (pharmacodynamics) (46–48).

Pharmacokinetic and pharmacodynamic end points are a critical component of contemporary drug development in the postgenome era, as researchers try to establish the best ways to use the new agents. It is only with such end points that researchers can follow the biochemical and biologic effects of a drug from simple model systems (e.g., enzyme assays, cancer cells in tissue culture) through animal testing (e.g., human xenografts and transgenic models) to clinical outcomes in patients. Pharmacokinetic and pharmacodynamic end points enable the assembly of a pharmacologic audit trail that can help address issues arising at all stages of the drug development process (46) (Fig. 4). An audit trail such as this documents the answers to a number of important questions that must be asked during development of a drug:

- *Is the target expressed and is the pathway active?* Answers to this question are helpful in selecting the best models to use in preclinical studies and which patients to include in clinical trials. It is valuable to understand the relationship among target expression, pathway activity, and response to the therapeutic agent.

- *Are sufficient concentrations achieved in plasma, blood, and tissues?* This information is critical, for if concentrations required for activity of the drug are not achieved in animal studies, or in patients, it is pointless to proceed further. Time and effort can then be focused on modifying the chemical structure of the drug to overcome the problem, or diverted to other projects if there is no way forward.
- *Is there activity on the desired molecular target?* For example, inhibition of a hypothetical kinase could be measured by assessing the phosphorylation of a downstream substrate. It is crucial to know the answer to this question, since lack of activity on the target is clearly a major reason for lack of therapeutic benefit.
- *Is there modulation of the desired biochemical pathway?* This question can be answered by using phosphospecific Abs to monitor the activation of downstream components of the pathway (e.g., phospho-ERK 1/2 in the mitogen-activated protein kinase [MAPK] pathway). It would also be valuable to assess the “off-target” effects on other signaling pathways, particularly those pathways of key therapeutic or toxicologic importance. To this end, gene expression microarrays and proteomic techniques can be used to profile changes to the transcriptome and proteome, respectively.
- *Have we achieved the desired biologic effect?* For example, do treated cells or tumors undergo apoptosis, necrosis, or cytostatic growth arrest? It is particularly important to distinguish between a cytostatic and cytotoxic tumor response for drugs in development, not least because cytostatic drugs will likely require continual administration to maintain tumor growth suppression, whereas more cytotoxic drugs will be best administered at intervals.
- *Is a clinical benefit seen?* Ultimately, this question can only be answered through the conduct of large, randomized phase 3 drug trials, which cannot be done for all drugs in development. Researchers need to short-list the most promising drugs based on surrogate end points of efficacy (e.g., time to progression or evidence of biologic response) to select compounds with the best likelihood of success.

## Imatinib Mesylate as a Paradigm for Cancer Therapy

Imatinib mesylate (STI571, see structure in Fig. 3) represents one of the most dramatic successes for the first wave of molecular therapeutics. It was discovered in the late 1980s by high-throughput screening, searching for compounds with kinase inhibitory activity. One of the hits found during the screen was a compound of the 2-phenylaminopyrimidine class; this compound had low potency and poor specificity but was the starting point for the synthesis of a number of improved analogs. Based on an iterative exploration of structure–activity relationships, this series of compounds was optimized to inhibit a variety of targets. Imatinib mesylate emerged as the lead compound from a series initially optimized against the platelet-derived growth factor receptor (PDGFR), based on its selective activity and drug-like pharmacokinetic properties. It was noted to be a potent inhibitor of BCR-ABL, a fusion protein kinase found in CML, and also of c-KIT, a tyrosine kinase found mutated in most cases of GIST (49).

### Imatinib Mesylate in CML

CML is a clonal hematopoietic stem cell disorder that accounts for approx 20% of all cases of leukemia. Clinically, the disease follows three distinct phases: chronic, accelerated, and blast. The chronic phase lasts for approx 5 yr and is characterized by an excess of normally differentiated myeloid cells; however, the disease subsequently transforms through an accelerated phase to an acute leukemia (blast crisis) that is invariably fatal.

Progression of CML through these three clinical phases can be characterized at the molecular level by an accumulation of abnormalities that eventually leave the cells

unable to differentiate normally. Chief among these abnormalities is the BCR-ABL fusion protein, formed by a reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11) (50–52). This abnormality can take several forms, depending on the breakpoint in BCR, but 95% of patients with CML have the p210 BCR-ABL form. BCR-ABL is crucial for the pathogenesis of CML, causing activation of a variety of intracellular-signaling pathways that lead to alterations in cell proliferation, adhesion, and survival. All of these events are dependent on the tyrosine kinase activity of the fusion protein. In addition, transduction of BCR-ABL into murine hematopoietic stem cells followed by transplantation into syngeneic mice causes a CML-like syndrome. Thus, BCR-ABL is an ideal target for treatment of the high proportion of patients who have the corresponding genetic abnormality.

Not surprisingly, imatinib mesylate was quick to enter phase 1 trials, initially in patients with chronic-phase CML who had failed therapy with interferon- $\alpha$  (IFN- $\alpha$ ). At doses >300 mg, 53 of 54 (98%) patients achieved a complete hematologic response, which was maintained in 51 of 53 (96%) patients. Doses at this level were extremely well tolerated, with side effects including nausea, vomiting, fluid retention, muscle cramps, arthralgias, and myelosuppression. The pharmacokinetics of imatinib mesylate are also good; its half-life of 13–16 h is sufficiently long to permit once-daily oral dosing (53).

With such impressive levels of activity in patients with chronic-phase CML who had failed IFN- $\alpha$ , the phase 1 studies were rapidly broadened to include patients with CML in accelerated phase and blast crisis, and also patients with relapsed or refractory, Philadelphia chromosome-positive acute lymphocytic leukemia (54). Response rates of 55% were obtained in both groups of patients; however, these responses tended not to be durable. Patients with myeloid blast crisis fared better, with 18% continuing on imatinib mesylate in remission up to 1 yr, but, unfortunately, nearly all of the patients with the lymphoid phenotype relapsed between 1 and 4 mo. Nonetheless, imatinib mesylate has remarkable single-agent activity in these difficult-to-treat patients.

Phase 2 studies tested imatinib mesylate further in patients with IFN-refractory, accelerated-phase, and myeloid blast crisis disease, confirming the pattern of response seen in the smaller phase 1 studies (55–66). The recommended dose was based on pharmacokinetic studies showing that a trough level of 1  $\mu\text{M}$  was reached at a dose level of 300 mg. This corresponded to the concentration needed for maximum cell kill in vivo, as well as the dose threshold for significant therapeutic benefits. The main outcome criteria used in these studies were hematologic response, cytogenetic response, and relapse rate at 18 mo. In patients with chronic-phase CML, the response rates were 95, 60, and 9%, respectively. In accelerated phase, it was 53, 26, and 40%, respectively. In myeloid blast crisis it was 29, 15, and 78%, respectively. These studies formed the basis for accelerated Food and Drug Administration (FDA) approval of imatinib mesylate in CML.

### ***Imatinib Mesylate in GIST***

Although originally derived from a screen for PDGFR tyrosine kinase inhibitors and studied in patients with BCR-ABL-driven CML, imatinib mesylate was subsequently shown to inhibit mutated c-KIT tyrosine kinase associated with GIST, a malignancy highly refractory to standard chemotherapies.

Clinical studies showed response rates close to 60% (67,68). Therapy was well tolerated, although mild edema, diarrhea, and fatigue were common. Imatinib mesylate was well absorbed, with pharmacokinetics similar to those seen in the CML studies.

Interestingly, patients with mutant c-KIT respond better than patients with the wild-type kinase. Imatinib has received regulatory approval for the treatment of GIST.

### **Resistance to Imatinib Mesylate**

Resistance to imatinib mesylate in CML can be classified as *de novo* resistance and relapse after an initial response. Most patients who relapse after an initial response to imatinib mesylate have reactivation of BCR-ABL, pointing to resistance mechanisms that either prevent imatinib mesylate from reaching the target, amplification of BCR-ABL, or mutations in BCR-ABL rendering the protein insensitive to the drug. The point mutations in imatinib mesylate-resistant cases are located in or adjacent to the adenosine triphosphatase (ATP)-binding site of the protein, and in the kinase activation loop. BCR-ABL mutation and amplification have not been commonly found in patients with *de novo* resistance (69,70).

Equally interesting are the mechanisms of resistance in patients with GIST. As previously discussed, imatinib mesylate exhibits good clinical response rates through its inhibition of the mutated c-KIT tyrosine kinase found in approx 85% cases of GIST. It has been shown that one third of patients with GIST lacking KIT mutations have activating mutations in the related receptor tyrosine kinase (RTK), PDGFR (71). Thus, KIT and PDGFR mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GIST.

### **Targeting ErbB Receptor Signaling**

ErbB receptors are a family of structurally related tyrosine kinase receptors that are important mediators of the proliferation, differentiation, and survival of normal cells (72). Four ErbB members have been identified: ErbB1 (also called HER1 or EGFR), ErbB2 (also called HER2 or neu), ErbB3 (HER3), and ErbB4 (HER4). Most therapeutic efforts to date have been focused on ErbB1 and ErbB2, hereafter referred to as epidermal growth factor receptor (EGFR) and HER2/neu, respectively.

The importance of ErbB receptors in cancer has long been recognized; they have been implicated in cellular proliferation, apoptosis, differentiation, angiogenesis, motility, and invasion (73). The dysregulation of ErbB function is known to occur by several mechanisms including gene amplification causing receptor overexpression and ErbB mutations that increase receptor activity. Preclinical studies have established that blocking ErbB receptor activity results in blockade of downstream signaling through the RAS/RAF/MEK/MAPK pathway (Fig. 1) and delayed tumor growth or tumor shrinkage in vivo.

As would be expected, ErbB signaling is not simply a linear pathway; extensive “cross talk” occurs between the four ErbB receptors and between the ErbB family and other transmembrane receptors (e.g., G-protein-coupled receptors and estrogen receptors [ERs]) (74). As well as inhibiting growth through the MAPK pathway, activation of ErbB signaling inhibits apoptosis through the activation of the phosphatidylinositol 3' kinase (PI3K) pathway (75) and alters cell motility, migration, and adhesion through its effects on focal adhesion kinase (76).

### **Targeting EGFR: Small Molecule or Antibody?**

The two most important strategies to target EGFR overactivity have been small-molecule inhibitors of the intracellular tyrosine kinase or monoclonal antibodies (MAbs) targeting the extracellular domain and inhibiting kinase activity indirectly. In addition, antisense and toxic immunoconjugates to ErbB1 are under investigation (77).



Each of the main approaches have advantages and disadvantages. Small-molecule inhibitors acting at the ATP-binding site of EGFR tyrosine kinase probably have better tumor penetration and the potential to inhibit the tyrosine kinase activity of other ErbB family members. On the other hand, it is argued that MABs not only block ErbB signaling, but also induce an anticancer cytotoxic immune response. This latter point is supported by evidence of greater antitumor effect *in vivo* than *in vitro*, but this may also be caused by antiangiogenic effects of the Ab.

### **Gefitinib**

Gefitinib or ZD1839 is a 4-anilinoquinazoline inhibitor of EGFR tyrosine kinase (structure in Fig. 3), with high specificity and potency ( $IC_{50}$  23 nM). It also inhibits downstream signaling, with increased p27<sup>Kip1</sup> and decreased activity of CDK2, thus inducing G1 cell-cycle arrest (78–81). Apoptosis is seen at higher doses, and it increases the proapoptotic effects of all cytotoxics evaluated on EGFR-overexpressing cells (77).

Gefitinib has excellent oral bioavailability, with little toxicity at active doses in mice, causing 50% tumor reduction at a daily oral dose of 10 mg/kg and complete responses at 200 mg/kg. Tumors usually regrew when gefitinib was stopped, however. In the presence of EGFR, low gefitinib concentrations also inhibit the growth of HER2/neu-overexpressing breast cancer cells, showing greater growth-inhibitory effects than trastuzumab (80).

In phase 1 clinical trials, gefitinib was given as a daily oral dose over 14 or 28 d. DLT were diarrhea and acneform skin rash, thought to be related to high EGFR expression in skin (82–84). Skin was proposed as a surrogate tissue for pharmacodynamic studies because of ease of access and high EGFR expression. These studies confirmed inhibition of EGFR signaling by immunohistochemistry (IHC) (decreased Ki-67, increased p27<sup>Kip1</sup>, increased phospho-STAT3).

Phase 2 studies showed promising clinical antitumor activity, with objective responses seen in patients with NSCLC, squamous cell carcinomas of head and neck, and hormone-refractory prostate cancer (85–89).

This preclinical and clinical progress led to two large, multicenter, randomized, controlled phase 3 trials of gefitinib in combination with cytotoxic chemotherapy. Chemotherapy (either gemcitabine/cisplatin [90] or paclitaxel/carboplatin [91]) was given with or without gefitinib. The trial's results showed that the addition of gefitinib made no difference in outcome (92). These results were somewhat surprising, given the evidence of activity using gefitinib as a single agent in NSCLC, head-and-neck malignancy, and hormone-refractory prostate cancer. One possible reason for the apparent lack of activity is that gefitinib targets the same tumor cell population as cytotoxic chemotherapy, thus potentially losing any opportunity for additive, let alone synergistic, effects. A second possibility is that if gefitinib causes cell-cycle arrest, it will antagonize the effects of cytotoxic therapy, which requires cycling cells. In addition, it may be possible to identify the molecular phenotype of a responsive subgroup that could be used to define who should receive the treatment in the future. This finding would be analogous to the FDA-approved use of HER2/neu status to decide which patients should receive trastuzumab (discussed later). On the basis of its earlier clinical success, gefitinib has received regulatory approval in the United States and Japan for its use as a single agent in the treatment of patients with NSCLC after failure of both platinum-based and docetaxel chemotherapies.

### **OSI-774**

OSI-774 is another small molecule with physicochemical and pharmacologic properties very similar to those of gefitinib (81). It is a highly specific, reversible, and

potent ATP-competitive inhibitor of EGFR tyrosine kinase ( $IC_{50}$  2 nM). In vitro studies showed that 50% inhibition of EGF-mediated autophosphorylation occurred at 20 nM, and that this caused 50% growth inhibition in HN5 head-and-neck tumor cells. In vivo studies using HN5 xenografts demonstrated that tumor shrinkage occurred with an oral dose of 100 mg/kg.

During phase 1 trials, OSI-774 was well tolerated with common toxicities again being diarrhea and acneform rash. On an uninterrupted, oral, daily dosing schedule, diarrhea was dose limiting, precluding escalation beyond 150 mg/d. At this dose, steady-state concentrations were approximately double the active concentrations seen in animal studies. Encouragingly, a number of partial responses have been reported in patients with metastatic renal and colorectal carcinomas and more patients with relatively long periods of stable disease (85,93–98). Phase 3 studies are under way in combination with cytotoxic chemotherapy.

### ***CI-1033, an Irreversible, Pan-ErbB Tyrosine Kinase Inhibitor***

CI-1033 is an orally active 4-anilinoquinazoline that acts as an irreversible pan-ErbB tyrosine kinase inhibitor. It has been developed after in vivo studies indicating that maximal antitumor activity occurs with prolonged inhibition of EGFR (99). CI-1033 suppresses the growth of human tumor xenografts, with the main side effect in animals being diarrhea.

Phase 1 studies have shown the drug to be generally well tolerated, with nausea, vomiting, fatigue, and diarrhea being the most common side effects in patients. Pre- and posttreatment tumor biopsies were taken for pharmacodynamic studies using IHC (100,101).

Irreversible inhibitors like CI-1033 could have the advantage of eliminating kinase activity until new receptors are made; however, this seems to happen rather quickly, probably within 2 d. Nevertheless, one would probably only need to maintain effective plasma concentrations of the drug for a shorter period compared to a reversible inhibitor. Targeting all four ErbB receptors may have the advantage of blocking redundant signaling that might be used to bypass more specific ErbB tyrosine kinase inhibitors; such agents could be more effective at preventing the emergence of drug resistance.

### ***Other Small-Molecule EGFR Tyrosine Kinase Inhibitors***

Other small-molecule EGFR tyrosine kinase inhibitors under development include WKB-569 (irreversible, EGFR + HER2/neu), PKI166 (EGFR), GW2016, and AG-1478 (HER2/neu).

### ***Anti-EGFR MABs***

The second prominent strategy for targeting EGFR tyrosine kinase has been to raise MABs to the extracellular-receptor domain, inducing a conformational change that prevents activation of tyrosine kinase and downstream signaling. In addition, Abs may induce an immune response.

### ***Cetuximab***

Cetuximab is a human-to-murine chimeric MAB that binds EGFR with an affinity 10 times that of the EGFR ligands, EGF, and transforming growth factor- $\alpha$ . It has been shown to block EGFR signaling, inducing p27<sup>Kip1</sup> expression, and arresting EGFR-expressing tumors in G1 (102).

Cetuximab inhibits the growth of a wide range of EGFR-expressing human tumor xenografts, causing shrinkage in some. It has been shown to enhance the activity of radiotherapy, cytotoxic chemotherapies, and MABs raised against HER2/neu (103–105).

In phase 1 clinical trials, cetuximab was given as a single weekly iv dose, and a dose of 200 mg/m<sup>2</sup> was considered optimal, based on saturation of clearance of the drug (106). Toxicity was minimal and included the now familiar acneform rash.

Phase 1 and 2 combination trials have been particularly interesting (77,107–109). Patients receiving cetuximab during a course of radiotherapy for locally advanced head-and-neck cancer achieved an overall response rate of 93% (109). In another study, patients with colorectal tumors with disease progressive on prior cisplatin-based or CPT-11-containing treatment were retreated with the same chemotherapy at the same dose, plus cetuximab. Seven of eight patients had a partial response (20). Phase 3 trials are ongoing (110).

### **ABX-EGF**

ABX-EGF is a fully humanized anti-EGFR MAb produced using the XenoMouse<sup>®</sup>, a transgenic mouse engineered to express human immunoglobulin genes; hence, it should be less immunogenic than mouse or chimeric Abs. ABX-EGF binds EGFR with high affinity, inhibits its tyrosine phosphorylation, and inhibits cell proliferation of EGFR-expressing human cancer cell lines.

Phase 1 studies showed the drug to be well tolerated at doses predicted to induce antitumor activity (>1.0 mg/kg), with a transient acneform rash reported. No human anti-human antibody responses have been reported to date (77). One phase 2 study used eight weekly infusions of ABX-EGF in patients with EGFR-positive metastatic renal cell carcinoma who either failed or could not receive therapy with interleukin-2 or IFN- $\alpha$  (111). Grade 2/3 toxicities included skin rash (60%), pruritus, dyspnea, fatigue, diarrhea, abdominal pain, and nausea and vomiting.

### **Targeting HER2/neu**

An important therapeutic strategy against HER2/neu is the use of Abs to the extracellular domain of the receptor, including trastuzumab, one of the most successful molecularly targeted agents to date. Other strategies under investigation include anti-HER2 Abs coupled to immunotoxins and cytotoxics, bispecific Abs binding both HER2/neu receptor and antitumor immune effector cells, straight immunization with HER2/neu protein, HER2/neu antisense, and targeted adenoviral gene therapy. In addition, several specific, small-molecule inhibitors of HER2/neu tyrosine kinase are under development (77).

### **Trastuzumab**

Trastuzumab is a humanized MAb with high affinity for HER2/neu ( $K_d = 0.1$  nM). It potently inhibits the proliferation of HER2/neu-overexpressing breast cancer cells in vitro, promoting accelerated HER2/neu internalization and degradation.

Three phase 1 clinical trials tested trastuzumab and were primarily designed to determine the safety and pharmacokinetics of trastuzumab (10–500 mg) administered intravenously as single or weekly doses. Treatment was well tolerated, with side effects including chills, asthenia, fever, and cardiotoxicity.

Phase 2 studies have examined the use of fixed-dose trastuzumab either as a single agent or in combination with cytotoxic chemotherapy. In one pivotal trial, single-

agent trastuzumab was administered to 222 patients with HER2/neu-positive metastatic breast cancer who had relapsed after one or two courses of chemotherapy (112). The overall response rate was 21% when assessed in evaluable patients by the investigators and 15% when analyzed on an intent-to-treat basis. Side effects commonly observed with chemotherapy, such as alopecia, mucositis, and neutropenia, were rarely seen.

Trials with trastuzumab in combination with chemotherapy were pursued based on evidence of synergy in xenograft studies. The cardiotoxicity seen occasionally with single-agent trastuzumab is significantly worse when the drug is given in combination with anthracycline-containing regimes (seen in 27% of those patients receiving doxorubicin/cyclophosphamide and trastuzumab [113]). Additional phase 2 studies have explored the effect of trastuzumab in a range of other cancers, including NSCLC and ovarian, bladder, and prostate cancer (115–122).

Phase 3 randomized controlled trials (113,122) showed that combination therapy with anthracycline- or taxane-based regimes significantly prolonged the median time to disease progression, increased the overall response rate, and increased the duration of response. Addition of trastuzumab improved the overall survival of patients with advanced metastatic breast cancer from 20.3 to 25.1 mo ( $p = 0.046$ ) (44). As with previous studies, the benefit of the addition of trastuzumab was particularly marked for patients whose tumors were strongly positive for HER2/neu.

Current studies are examining whether trastuzumab can be administered intravenously every 3 wk or by the sc route. If successful, these regimens would reduce the number of hospital visits, ease administration of the drug, and potentially prove more cost-effective. Furthermore, various combinations of trastuzumab and chemotherapeutic agents are being explored with the aim of identifying the optimal combination regimen for clinical use.

### ***Future Challenges for Blockade of ErbB Signaling***

Blockade of ErbB tyrosine kinase and downstream signaling has produced encouraging results with a first wave of molecular therapeutics now in advanced clinical trials. Because preclinical studies indicate that tumor regrowth occurs once ErbB-targeted therapies are stopped, the safety of long-term continuous administration must be assessed, as must the optimal dosage, long-term toxicity, and mechanisms of drug resistance.

Also important will be determining which patients should receive treatment. To date, the results with trastuzumab indicate that the patients who benefit most are those with the highest levels of HER2 expression. Overexpression does not necessarily imply overactivity, and assays of both receptor and pathway activation will be helpful to interpret the efficacy seen in patients. Unlike trastuzumab and HER2/neu, no simple correlation exists between sensitivity to EGFR tyrosine kinase inhibitors and EGFR expression in cell lines or xenografts (123). Thus, functional assessment of EGFR autophosphorylation and effects on downstream ERK 1/2 and AKT phosphorylation may be useful in selecting those patients most likely to benefit from therapy.

With respect to ErbB3 and ErbB4, specific agents targeting these receptors are not as advanced in their development for at least two reasons. First, ErbB3 lacks an intracellular-signaling domain and is dependent on other ErbB members for its activation.

Second, both receptors have been associated with ER expression and good prognosis in metastatic breast cancer, which questions their relevance as therapeutic targets.

### **Retinoids: Targeting the PML-RAR Fusion Protein in APL**

APL is a distinct subtype of acute myeloid leukemia (AML) in which a balanced reciprocal translocation between chromosomes 15 and 17 results in the formation of a chimeric gene that encodes the formation of the PML-RAR $\alpha$  fusion protein. In normal mammalian cells, the PML protein is primarily localized in multiprotein nuclear complexes called PML nuclear bodies. Indeed, PML protein is a negative growth regulator capable of causing growth arrest in G1 of the cell cycle, transformation suppression, senescence, and apoptosis. The fusion protein PML-RAR $\alpha$  causes dramatic deregulation of growth control and has a crucial importance in driving and maintaining malignant progression in APL.

Although most patients with APL achieve a complete remission with anthracycline-based chemotherapy regimes, up to 20% will have a terrible hemorrhagic syndrome either before or during chemotherapy. This bleeding disorder is attributed to disseminated intravascular coagulation, fibrinolysis, and proteolysis.

The introduction of ATRA in patients with newly diagnosed APL, either alone or combined with chemotherapy in induction, has improved overall survival up to 80% (25,124–127). ATRA works by differentiating leukemic promyelocytes into mature cells. The European APL Group compared therapy with ATRA plus chemotherapy vs concurrent ATRA until complete remission, and both cohorts then received further chemotherapy. The group found a significantly reduced relapse rate at 2 yr among those patients receiving concurrent therapy (6 vs 16%;  $p = 0.04$ ) (127).

### **Farnesyl Transferase Inhibitors**

An alternative potential strategy to tackle the dysregulated receptor tyrosine kinase/RAS/MEK/MAPK pathway is to target RAS. The RAS proteins were some of the first proteins identified that possessed the ability to regulate cell growth, and they are often constitutively active in cancer because of point mutations in *RAS* genes (128). More important, approx 20% of all tumors have an activating mutation in one of the *RAS* genes (129). In these tumors, mutant *RAS* drives several aspects of the malignant phenotype, including the deregulation of cell growth, apoptosis, and angiogenesis. Despite some success with anti-RAS antisense therapy and agents designed to block RAS in the inactivated state, these approaches are practically difficult. The development of FTIs was based on the supposition that inhibition of posttranslational modification and membrane localization of RAS would block proliferative signal transduction (130).

Some debate exists about the precise mechanism of action of FTIs. In fact, they may not be exerting their antitumor effects through the farnesylation of *RAS* but possibly through effects on the prenylation of other proteins, such as RhoB, an endosomal protein that has been implicated in selective growth inhibition and apoptosis in neoplastic cells (131).

Nonetheless, a number of FTIs have progressed through clinical trials. Of these, the use of the methylquinolone R115777 and the tricyclic SCH66336 as single agents have demonstrated disease stabilization or objective responses in 10–15% of patients with refractory malignancies.

### **R115777**

R115777 (Zanestra, see structure in Fig. 3) was shown to have antiproliferative effects on human tumor cells in vitro ( $IC_{50}$  in the submicromolar range) and in vivo (significant tumor growth inhibition at 25 mg/kg and above). In vivo tumor response was correlated with an increase in the amounts of prelamin A in both tumor tissue and peripheral blood lymphocytes.

In phase 1 trials, DLT involved a combination of myelosuppression, fatigue, and peripheral neuropathy at a dose of 400 mg twice daily, and plasma levels of R115777 at the MTD clearly exceeded those that were active in vitro (132–136).

In patients with solid tumors, accessible normal tissues such as peripheral blood lymphocytes and buccal mucosa have provided surrogate tissues that allow confirmation that farnesyltransferase is inhibited in vivo at clinically achievable drug doses.

After hints of clinical activity in patients with pancreatic, colon, and NSCLC, a number of site-specific phase 2 trials were completed, which confirmed significant antitumor efficacy in 6 of 20 (30%) patients with refractory acute leukemia and 3 of 27 (11%) patients with advanced breast cancer (137–144).

One multicenter, randomized, placebo-controlled phase 3 trial of R115777 has been reported in patients with metastatic colorectal cancer (145). R115777 failed to show a significant increase in overall survival compared with placebo; median overall survival was 5.7 and 6.1 mo, respectively ( $p = 0.396$ ). Another trial comparing gemcitabine with either R115777 or placebo in patients with advanced pancreatic cancer also showed no difference in overall survival (146).

### **SCH66336**

A second compound, SCH66336 (Sarasar), has shown encouraging results in clinical trials. Using either a 1-wk on, 3-wk off or a 2-wk on, 2-wk off schedule, an MTD of 400 mg twice daily was reached, with DLT being myelosuppression, diarrhea, vomiting, and nephrotoxicity (147,148).

Adjei et al. (149) reviewed the pharmacodynamic markers used in the different FTI trials, and tested them against a range of human cell lines in vitro. These included measurement of farnesyltransferase enzymatic activity, and measurement of accumulation of p21<sup>Waf1</sup> after treatment with FTI. The most consistent changes seen in these experiments were increases in the chaperone protein HDJ-2 and prelamin A (a nuclear protein that normally undergoes farnesylation). Use of these pharmacodynamic markers will be helpful in the selection of the optimum dose and schedule to inhibit protein farnesylation in vivo.

### **RAF and MEK Inhibitors**

It has been technically difficult to inhibit the *RAS* gene or its protein product directly, and inhibition of farnesyltransferase has been more complex than originally thought (150). An alternative approach to the pathologic signaling through the MAPK pathway is to inhibit downstream RAF or MEK. Because both of these are kinases, they are much more “drug-able” targets.

The leading RAF kinase inhibitor, BAY 43-9006 (structure in Fig. 3), has exhibited good antiproliferative properties in vitro and in vivo (151). It has shown hints of activity in phase 1 trials and is currently in phase 2 trials.

Moving further down the signaling pathway, the MEK inhibitor CI-1040 (structure in Fig. 3) has also passed through successful preclinical studies (152) into phase 1 clinical trials, which have used inhibition of phospho-ERK in patient peripheral blood lymphocytes as a pharmacodynamic marker. It is also in phase 2 studies.

### ***Inhibitors of the PI3K Pathway***

The PI3K/AKT/mTOR pathway has, in recent years, been recognized as an important regulator of cell proliferation and survival (Fig. 1). Gain- or loss-of-function mutants of several components of the pathway lead to neoplastic transformation in model systems, and many components of the PI3K-AKT pathway are deregulated in a wide range of human cancers (153,154). For example, *PTEN*, the most highly mutated tumor suppressor gene (TSG) in cancers after *p53*, acts as a phosphatase that hydrolyzes the activated inositol lipid product of the PI3K reaction (phosphatidylinositol 3,4,5'-triphosphate), antagonizing the action of PI3K. This gene is mutated or silenced in glioblastomas, as well as colorectal, lung, and endometrial carcinomas among others.

Prototype inhibitors of PI3K, such as the flavone LY 294002 and the natural product wortmannin, have shown anticancer activity in vitro and in animal models. Although widely used as experimental tools, these agents have limitations in terms of their drug-like properties. Wortmannin is a potent inhibitor but is rather unstable, whereas LY 294002 is less potent and has metabolic liabilities. In addition, both agents show broad activity across the many members of the PI3K superfamily. Evidence suggests that the p110 $\alpha$  isoform is likely to be the preferred target in cancer, and potent and selective inhibitors of this isoform would be of great interest.

The clinical evidence of PI3K deregulation in human cancers and the identification of downstream kinases on the pathway (e.g., AKT, mTOR, PDK1, and ILK) provide a series of potential targets for the development of small-molecule therapies.

### ***Inhibitors of mTOR***

RAD001 is an oral macrolide analog of rapamycin that targets mTOR to inhibit the downstream signaling events involved in the regulation of G1- to S-phase transition. The main downstream targets of mTOR are p70 S6 kinase and the eIF-4E-binding protein (4E-BP1). RAD001 has demonstrated in vitro and in vivo antiproliferative activity against a number of human tumor cell lines (155). In vivo studies have shown that a single administration of RAD (5 mg/kg) causes significant inactivation of p70 S6 kinase in tumor biopsies, skin biopsies, and peripheral blood lymphocytes. In pre-clinical models, significant inhibition of tumor p70 S6 kinase was maintained up to 48 h, and inhibition over 72 h was seen in skin and peripheral lymphocytes (155,156). In another study, it was noted that a human cancer cell line (the B16/BL6 melanoma model) was more sensitive in vivo than in vitro. Tumor-associated blood vessel density was decreased >50% after treatment with RAD001, suggesting additional antiangiogenic effects that might explain the difference in sensitivity. These observations provide in vivo validation for using the inhibition of p70 S6 kinase inhibition in tumor, skin, or lymphocytes as a pharmacodynamic marker in clinical trials. Phase 1 studies are under way.

CCI-779 is a second inhibitor of mTOR, which is advanced in its clinical development. After showing evidence of activity in phase 1 trials, including a partial response

in a patient with breast cancer, recently reported ongoing phase 2 studies have confirmed signs of activity in breast cancer, and renal cell cancers (157,158).

Use of mTOR inhibitors is more effective against cell lines that have an activated PI3K pathway (159), a finding that may have implications for the choice of patients who may benefit from treatment with these agents.

### Cell-Cycle Inhibitors

A number of signal transduction pathways converge on the cell-cycle control apparatus, which has become a major area of targeted therapeutic research. Cyclin-dependent kinases (CDKs) are frequently deregulated in cancer by several means and represent an exciting area of targeted drug development (see also Chapter 11).

Flavopiridol is a relatively broadly acting CDK inhibitor (CKI) that is in phase 2 clinical trials. The R-enantiomer of the purine roscovitine (CYC202, see structure in Fig. 3) is a relatively selective inhibitor of CDK2 that is completing phase 1 studies. Staurosporine is a broadly acting kinase inhibitor, but its 7-hydroxy analog shows a high degree of selectivity for the checkpoint kinase CHK1 and is now in phase 2. Numerous other members of the cell-cycle and checkpoint control machinery represent important potential therapeutic targets (160). As with other mechanism-based agents, the use of pharmacokinetic–pharmacodynamic end points will be extremely important. For example, inhibition of Rb phosphorylation can provide a useful measure of the action of a CKI. A number of newer CKIs are emerging with the potential for selectivity against particular CDKs, such as PD 0183812 (161), which inhibits CDK4 and CDK6, and BMS 387032, which is a representative of a potent class of CDK1 and CDK2 inhibitors (162). It will be interesting to see how these newer agents fare in clinical trials.

### Inhibitors of HSP90

HSP90 is a molecular chaperone that functions to stabilize several mutated and overexpressed “client” signaling proteins that promote the proliferation and survival of cancer cells (163,164). Prominent among the HSP90 client proteins are a number of oncogenic kinases, including RAF-1 (165), ErbB2 (166,167), CDK4 (168), Polo-1, and Met. Other client proteins relevant to the development and maintenance of cancer include mutant p53 (169), ER and androgen receptors (170–173), as well as AKT and the catalytic component of telomerase hTERT (163).

Inhibition of HSP90 leads to incorrect folding and subsequent degradation of oncogenic client proteins through the ubiquitin proteasome pathway. Inhibition of HSP90 is an extremely attractive means for tackling multiple deregulated genes and pathways, simultaneously killing many oncogenic birds with one stone. In this way, HSP90 inhibitors could have the potential to tackle all six of the so-called hallmark traits of malignancy (8,163).

In the early 1990s, certain natural products (the benzoquinone ansamycins in general, and geldanamycin in particular) were found to bind to HSP90. These agents compete with ATP for binding at the nucleotide-docking site located in the N-terminal domain of HSP90 (174,175). In doing so, they prevent the correct assembly of mature HSP90/client protein/cochaperone complexes, which leads to proteasomal degradation by recruitment of an ubiquitin ligase.

Geldanamycin proved too hepatotoxic for clinical use, but one of its derivatives, 17AAG, was found to be less hepatotoxic while retaining the antitumor activity of



geldanamycin (176). Various techniques including Western blotting and gene expression microarray analysis have been used to define a molecular signature of HSP90 inhibition (177), including the depletion of client proteins such as RAF-1, ErbB2, and CDK4 and the simultaneous upregulation of HSP70. Proteomics techniques were used successfully to identify a further marker, AHA1, which represents the first HSP90 cochaperone to be identified that acts as an activator of the ATPase activity of the chaperone (178).

17AAG was found to have good xenograft activity (179–181) and acceptable toxicity, and is currently in phase 1 clinical trials. Of particular interest in the phase 1 trial of 17AAG at our own center has been the use of a molecular signature to help define the optimal dose and schedule of the drug. The molecular signature was defined as reduced expression of RAF-1, CDK4, and LCK and increased expression of HSP70. This pattern of HSP90 inhibition has been followed and validated through cancer cells in culture and human xenografts to patient samples that include both peripheral blood lymphocytes and tumor biopsies (182). In addition, two patients have had stable disease after treatment with 17AAG for several months; in one of these patients, the rapid progression of her melanoma arrested quite dramatically. These results have stimulated interest in phase 2 studies, which are due to start shortly.

Despite the promising results with 17AAG, it does have limitations. For example, it is metabolized by both polymorphic cytochrome P450 and the quinone reductase DT-diaphorase and likely to be a substrate for P-glycoprotein (179). It also has relatively poor solubility, requiring a complex formulation that makes administration to patients rather cumbersome. Alternative HSP90 inhibitors have been identified, including novabiocin and the synthetic purine PU3 (163).

## Targeting Single vs Multiple Signal Transduction Pathways

The oncogene addiction hypothesis (183,184) emphasizes the importance of oncogenes or tumor suppressor genes (TSGs) in the maintenance and initial development of cancer. It is an important concept because it provides a potential means to explain how correcting a single key oncogenic lesion in a cancer that has undergone a number of activating oncogenic events may nevertheless provide an anticancer effect. Furthermore, oncogene addiction may explain how inhibitors of oncogenic signal transduction can exhibit selective effects in tumor vs normal cells. Elegant conditional expression studies were done in transgenic mice showing that expression of c-MYC led to the development of an aggressive sarcoma. When c-MYC expression was turned off, the malignant cells differentiated into normal bone. Furthermore, restoration of c-MYC expression resulted in apoptosis of the osteocytes rather than reversion to malignancy (196). A number of other experimental studies suggest that correction of a single oncogenic defect can produce an important anticancer effect, even in the presence of multiple oncogenic abnormalities. The thesis is that malignant cells have become physiologically dependent on continued activity of specific activated or overexpressed oncogenes for maintenance of the malignant phenotype.

It is likely that the multistage process of carcinogenesis is not just a simple summation of the individual effects of oncogene activation and tumor suppressor repression. The molecular circuitry of cancer that regulates signal transduction and gene expression is likely to be very different compared with that of normal cells. Only when researchers have a more detailed understanding of the deranged dynamic circuitry of

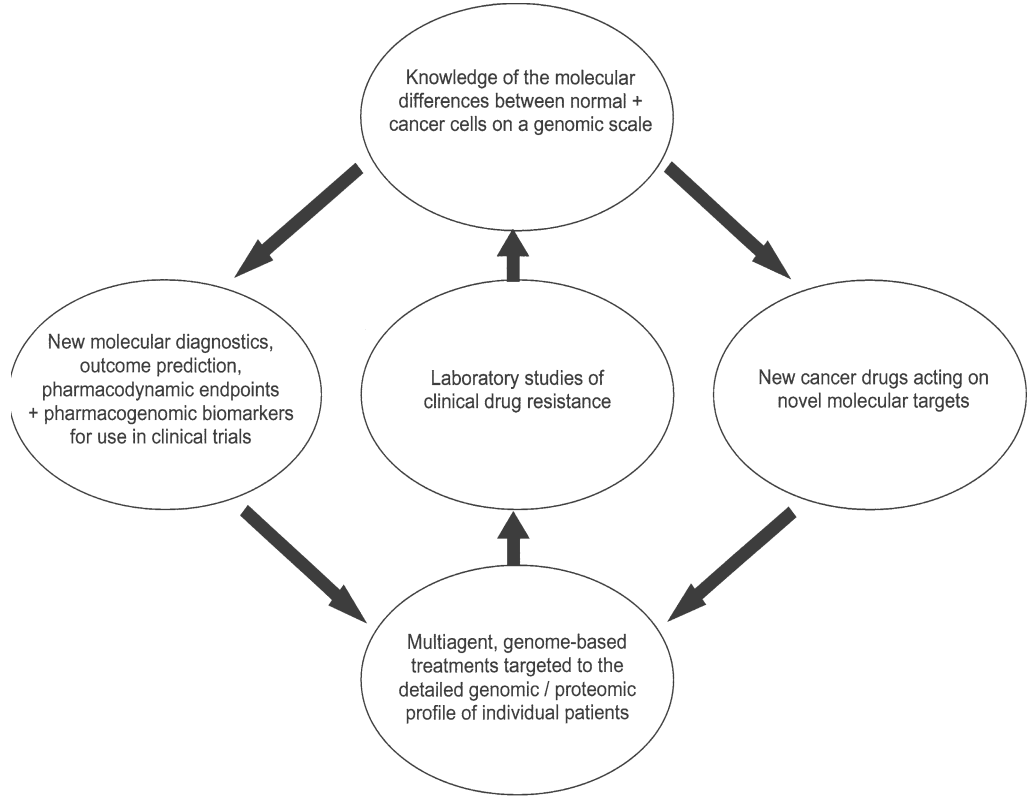


Fig. 5. Translational research in molecular therapeutics.

cancer cells will they be able to pinpoint with certainty which signaling pathways or nodes are the crucial ones to tackle for each cancer.

Nonetheless, the clinical exploitation of the oncogene addiction hypothesis is supported, e.g., by activity of imatinib against BCR-ABL in CML and ATRA against PML-RAR $\alpha$  in APL. Oncogene addiction can help explain why a selective anticancer effect can be obtained with molecular therapeutics that hit signal transduction pathways activated in cancer cells but that are required by normal cells. For example, mTOR inhibitors (e.g., CCI-779) and PI3K inhibitors (e.g., LY294002) have been shown to have potent activity against cancer cells that have lost PTEN tumor suppression (159).

CML, APL, and c-KIT mutant GIST are diseases that appear to be driven mainly by a single genetic defect, whereas most solid cancers are the result of the accumulation of numerous genetic abnormalities arising from disruption on a genomic scale. Although oncogene addiction and TSG hypersensitivity are still likely to apply to such tumors, it seems likely that multiple lesions will be involved in driving malignancy, leading to a degree of redundancy in oncogene signal transduction pathways. This probably explains why, despite having such impressive activity in the earlier, chronic phase of CML, imatinib mesylate produces only temporary responses in the

later accelerated and blast crisis stages of disease (55,58,59,64) and also in AML, in which other oncogenic lesions in addition to BCR-ABL are important for driving malignancy.

The significant but limited activity seen with agents such as trastuzumab and gefitinib may also be explained by the need to modulate multiple important oncogenic pathways in many solid tumors. For most human cancers, it seems unlikely that correction of a single molecular defect will be sufficient to achieve real clinical benefit, and that a combination of agents will be required. Cocktails of two or more signal transduction inhibitors have been shown to have additive antitumor effects; for example, the combination of a tyrosine kinase inhibitor with a COX-2 inhibitor was highly effective in an *in vivo* tumor model (185). In the short to medium term, however, the new molecular therapeutics are likely to find their place alongside traditional cytotoxic chemotherapies. Drugs that modulate targets that affect multiple features of the malignant phenotype, e.g., inhibitors of the proteasome, chromatin modifying enzymes such as histone deacetylases, and the molecular chaperone HSP90 will also play an important role.

## Conclusion

Proof of principle has now been established that targeting signal transduction pathways can be clinically beneficial. Tackling multistep carcinogenesis will most likely require combinatorial therapies; probably cytotoxic + signal transduction inhibitor combinations for the next 5–10 yr, or cocktails of signal transduction inhibitors in the future. The sophistication of our therapies will need to increase to match the intricacies of the disease. The long-term goal is to create effective and less toxic therapies matched to the specific molecular pathology of each individual patient.

The sequencing of the human genome together with the Cancer Genome Project and associated activities will accelerate the discovery and validation of new molecular targets for cancer drug development, as in the example of BRAF. The use of global gene expression and proteomic profiling will also be of major importance.

The process of drug discovery and development can be carried out quicker and more efficiently than before through a twofold strategy of focusing on important molecular targets and using modern high-throughput technologies to complement more traditional hypothesis-driven research. High throughput screenings, combinatorial chemistry, and other technologies are also accelerating the pace of drug discovery. Clinical trials of molecular therapeutic agents will increasingly involve early hypothesis testing, pharmacokinetic and pharmacodynamic end points, and careful selection of patients for treatment based on molecular pathologic criteria.

Cancer drug development is a multidisciplinary process whose success depends on the close collaboration among several disciplines including molecular biology, chemistry, pharmacology, and medicine. Figure 5 illustrates how effective translational research can be fostered by the rapid flow of information back and forth between laboratory and clinic. Table 6 lists some useful Websites covering the areas of genomics, signal transduction, and drug development.

The next few years will be very exciting, as new and more effective molecular therapeutics are discovered through a combination of the rational exploitation of our molecular understanding of cancer combined with a pragmatic, realistic approach to preclinical and clinical drug development.

**Table 6**  
**Useful Websites**

Topic	Site	Description
Human genome	<a href="http://www.ensembl.org/Homo_sapiens/">www.ensembl.org/Homo_sapiens/</a>	Ensembl Human Genome Browser; European Bioinformatics Institute (EBI), Sanger Centre, Hinxton, UK
	<a href="http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi">www.ncbi.nlm.nih.gov/mapview/map_search.cgi</a>	National Center for Biotechnology Information (NCBI) Human Genome Browser; National Institutes of Health (NIH), Bethesda, MD
	<a href="http://genome.ucsc.edu/cgi-bin/hgTracks">http://genome.ucsc.edu/cgi-bin/hgTracks</a>	University of California Santa Cruz (UCSC) Human Genome Browser; Santa Cruz, CA
	<a href="http://www.celera.com">www.celera.com</a>	Genome-sequencing company; Rockville, MD
	<a href="http://www.ncbi.nlm.nih.gov/SNP/">www.ncbi.nlm.nih.gov/SNP/</a>	Database of human Single Nucleotide Polymorphisms (dbSNP); NCBI, NIH, Bethesda, MD
Cancer genome	<a href="http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Unigene">www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Unigene</a>	Unigene database for nonredundant, gene-oriented clusters; NCBI, NIH, Bethesda, MD
	<a href="http://cgap.nci.nih.gov/">http://cgap.nci.nih.gov/</a>	Cancer Genome Anatomy Project; National Cancer Institute (NCI), NIH, Bethesda, MD
Genomics resources	<a href="http://www.nature.com/genomics/">www.nature.com/genomics/</a>	Nature Genome Gateway; Excellent resource of free original research papers, news + links to other sites
	<a href="http://www.sciencemag.org/feature/plus/sfg/">www.sciencemag.org/feature/plus/sfg/</a>	Science Functional Genomics; Similar quality + scope of the Nature Genome Gateway site
	<a href="http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books">www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Books</a> <a href="http://www.nature.com/genomics/">www.nature.com/genomics/</a>	The NCBI Handbook; NCBI, NIH, Bethesda, MD Users Guide to the Human Genome; Wolfsberg TG, et al. A user's guide to the human genome. <i>Nat. Genet.</i> (2002)
Bioinformatics resources	<a href="http://www.ebi.ac.uk/services/index.html">www.ebi.ac.uk/services/index.html</a>	Genomic, proteomic, structural databases + bioinformatics tools; European Bioinformatics Institute, Sanger Centre, Hinxton, UK
	<a href="http://www.hgmp.mrc.ac.uk/">www.hgmp.mrc.ac.uk/</a>	Human Genome Mapping Project Resource Centre (HGMP-RC); Genome Campus, Sanger Centre, Hinxton, UK
	<a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a>	National Center for Biotechnology Information; National Institutes of Health, Bethesda, MD
	<a href="http://www.nature.com/nrc/journal/v2/n5/weinberg_poster">www.nature.com/nrc/journal/v2/n5/weinberg_poster</a>	Subway map of cancer pathways (see ref. 193); Links to summaries of key proteins, cellular processes, and PubMed references
Signal transduction	<a href="http://cgap.nci.nih.gov/Pathways">http://cgap.nci.nih.gov/Pathways</a>	Cancer Genome Anatomy Project; BioCarta + KEGG pathways; Link from pathways to genes, proteins + expression in different tissues
	<a href="http://www.nature.com/nrm/FOCUS/signalling/">www.nature.com/nrm/FOCUS/signalling/</a>	Nature—Focus on Signalling; Articles from <i>Nature Reviews</i> series

	<a href="http://www.cellsignal.com/">www.cellsignal.com/</a>	Cell Signaling Technology Inc.; Produces reagents (e.g., phosphoantibodies) to study signal transduction
	<a href="http://www.ebi.ac.uk/microarray/">www.ebi.ac.uk/microarray/</a>	European Bioinformatics Institute; Tools for managing, storing, and analyzing microarray data
	<a href="http://www.crukdmf.icr.ac.uk/">www.crukdmf.icr.ac.uk/</a>	Cancer Research UK DNA Microarray Facility; Institute of Cancer Research, Sutton, UK
DNA microarrays and gene expression	<a href="http://www.affymetrix.com/index.affx">www.affymetrix.com/index.affx</a>	Affymetrix; Leading producer of oligo-gene expression microarrays
	<a href="http://www.microarrays.org/">www.microarrays.org/</a>	DeRisi Lab; University of California at San Francisco (UCSF)
	<a href="http://brownlab.stanford.edu">http://brownlab.stanford.edu</a>	Brown Lab; Howard Hughes Medical Institute, Stanford University, Standord, CA
Drug development	<a href="http://www-genome.wi.mit.edu/cancer/index.html">www-genome.wi.mit.edu/cancer/index.html</a>	Golub Lab; Center for Genome Research, Whitehead Institute, Stanford, CA
	<a href="http://discover.nci.nih.gov/">http://discover.nci.nih.gov/</a>	Weinstein Lab; Laboratory of Molecular Pharmacology, NCI, Bethesda, MD
	<a href="http://www.icr.ac.uk/cctherap/">www.icr.ac.uk/cctherap/</a>	Cancer Research UK Centre for Cancer Therapeutics; Institute of Cancer Research, Sutton, UK
	<a href="http://dtp.nci.nih.gov/">http://dtp.nci.nih.gov/</a>	Developmental Therapeutics Program; NCI, NIH, Bethesda, MD
	<a href="http://www.phrma.org/">www.phrma.org/</a>	Pharmaceutical Research and Manufacturers of America (PhRMA); US pharmaceutical and biotech companies
	<a href="http://www.nature.com/nrd/">www.nature.com/nrd/</a>	Nature Reviews Drug Discovery; Timely reviews from Nature Publishing Group
Pharmacogenetics and pharmacogenomics	<a href="http://www.pharmgkb.org">www.pharmgkb.org</a>	The Pharmacogenetics and Pharmacogenomics Knowledge Base; Stanford University, Stanford, CA
	<a href="http://www.nature.com/cgi-taf/dynapage.taf?file=/nbt/journal/v16/n2s/index.html">www.nature.com/cgi-taf/dynapage.taf?file=/nbt/journal/v16/n2s/index.html</a>	Nature Biotechnology Pharmacogenomics Supplement; Useful starting point for exploration of contemporary pharmacogenomics
Clinical trials	<a href="http://clinicaltrials.gov/">http://clinicaltrials.gov/</a>	Clinical Trials—site for public and professionals; NIH, Bethesda, MD
	<a href="http://ctep.info.nih.gov/index.html">http://ctep.info.nih.gov/index.html</a>	Cancer Therapy Evaluation Program; NCI, NIH, Bethesda, MD
	<a href="http://www.eortc.be">www.eortc.be</a>	European Organisation for Research and Treatment of Cancer (EORTC); Helps develop, conduct, and coordinate laboratory and clinical research in Europe
	<a href="http://ctep.info.nih.gov/reporting/ctc.html">http://ctep.info.nih.gov/reporting/ctc.html</a>	Common Toxicity Criteria; Standard terminology to name and grade severity of adverse events
Regulatory bodies	<a href="http://www.fda.gov/cder/">www.fda.gov/cder/</a>	Center for Drug Evaluation and Research; Food and Drug Administration
	<a href="http://www.emea.eu.int/">www.emea.eu.int/</a>	The European Agency for the Evaluation of Medicinal Products; Agency coordinating the regulatory policies of european states
	<a href="http://www.mca.gov.uk/">www.mca.gov.uk/</a>	Medicines and Healthcare products Regulatory Agency; UK regulatory body
	<a href="http://www.fda.gov/cder/cancer/druglistframe.htm">www.fda.gov/cder/cancer/druglistframe.htm</a>	List of FDA-approved oncology drugs; Lists drug, approved use, manufacturer, and approval date

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# 19

## Treatment of Carcinogenesis

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### Introduction

During the past several decades, cancer research has primarily focused on development of cytotoxic agents for treatments. These efforts have significantly improved the prognosis of some types of malignancies including some leukemias, lymphomas, and testicular carcinoma. However, other tumors including metastatic colorectal, breast, and lung carcinomas are still associated with poor prognosis. Innovative approaches to understanding the cellular and molecular mechanisms of the process of carcinogenesis have provided new insights into the paradigm of cancer treatment by exploring the possibilities of early detection, chemoprevention, and treatment of premalignant disease.

Intraepithelial neoplasia (IEN) is characterized as moderate-to-severe dysplasia that occurs on the causal pathway from normal tissue to malignancy. Accumulation of genetic mutations manifests in the phenotypic changes associated with cancer, including loss of cell-cycle control and apoptosis. Because the process of carcinogenesis often requires many years, identification of IEN at early stages affords numerous opportunities for intervention of the malignant progression.

Prevention of cancer through development of chemopreventive agents for high-risk populations and interventions for early stage carcinogenesis are key strategies of current oncology research. Research in the fields of chemoprevention and treatment of carcinogenesis has led to identification of diagnostic methodologies and potential agents that reduce cancer risk. Techniques that show promise for early diagnosis of cancer and potential agents for chemoprevention in high-risk populations and treatment of the early stages of carcinogenesis are being investigated in clinical studies to determine their ability to reduce cancer risk, morbidity, and mortality in selected populations. Table 1 summarizes some of the methods undergoing clinical study for early detection of cancers. Table 2 shows clinical studies designed to test chemopreventive agents for primary and secondary cancers. Of paramount importance to cancer research is elucidation of molecular targets and signal transduction pathways that regulate cel-

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**Table 1**  
**NCI Sponsored Clinical Studies Evaluating Methods of Early Cancer Detection**

Tumor type	Study title
Breast	Screening and diagnostic trial to study the effectiveness of MRI scans in women who are at high risk for developing breast cancer.
	Screening trial to compare specific biomarkers based on fine-needle aspiration specimens from women at increased or normal risk of breast cancer.
	Study to evaluate the rate of recurrence of breast cancer in women who have had surgery for ductal carcinoma in situ.
	Screening and diagnostic trial to compare the effectiveness of two types of mammography in detecting breast cancer in women
Cervical	Screening trial to compare different types of screening tests used to detect cervical neoplasia.
Colon	Randomized screening trial to compare the effectiveness of fecal occult blood testing to DNA-based testing of stool and blood in identifying colorectal cancer.
	Diagnostic and screening trial to compare the effectiveness of barium enema, computed tomographic colonography, and colonoscopy in detecting of colon cancer.
Genetic predisposition	Examination of clinical and laboratory abnormalities in patients with defective DNA repair: xeroderma pigmentosum, Cockayne syndrome, or trichothiodystrophy
Lung	Randomized clinical trial to compare computed tomography with chest radiograph in screening individuals who are at high risk for lung cancer.
	A pilot study to evaluate fluorescent light bronchoscopy plus conventional bronchoscopy as a tool for screening and detecting lung cancer in persons with completely resected head and neck cancer or successfully treated early-stage lung cancer.
	Screening and diagnostic study of computed tomography in women who are at risk for lung cancer.
Ovarian	Clinical trial to determine effective methods of identifying women who are at increased risk for developing ovarian cancer.
	Screening trial to determine the significance of CA 125 levels in detecting ovarian cancer in participants who have a high genetic risk of developing ovarian cancer.
	Screening trial to determine the best procedure to detect ovarian cancer in women who have a high genetic risk for developing ovarian cancer.

lular proliferation, differentiation, and apoptosis. These processes include signaling proteins such as cytokines, growth factors, adhesion molecules, and transcription factors that play key roles in regulation of growth and invasion of cancer cells. The concept of the treatment of carcinogenesis is dependent on several factors including development of detection methods for early stage carcinogenesis when prognosis is favorable and identification of novel agents with a favorable toxicity profile that could specifically target each stage of carcinogenesis.

This chapter discusses advances in our understanding of the cellular and molecular mechanisms of the multistep process of carcinogenesis, specific types of malignancies and the respective precursor lesions with which they are associated, and agents that have shown promise in preclinical and clinical investigation for chemoprevention and treatment of early stage neoplastic changes. Molecular targets for therapies, early diagnosis, and new diagnostic techniques are discussed.



**Table 2**  
**NCI-Sponsored Primary and Secondary Prevention of Cancer Studies**

Tumor type	Study title	Agent
Bladder	Randomized phase 3 trial to study the effectiveness of fenretinide in treating patients who are at risk for recurrent bladder cancer following surgery to remove the tumor	Fenretinide
	Randomized phase 2/3 trial to study the effectiveness of celecoxib in preventing disease recurrence in patients who have bladder cancer.	Celecoxib
Breast	Randomized double-blinded clinical trial to compare the effectiveness of raloxifene with that of tamoxifen in preventing breast cancer in postmenopausal women.	Raloxifene versus tamoxifen
	Phase 1 trial to study the effectiveness of indole-3-carbinol in preventing breast cancer in nonsmoking women who are at high risk for breast cancer.	Indole-3-carbinol
	Randomized pilot study to study the effectiveness of combining raloxifene and goserelin in preventing breast cancer in women who have a family history of breast cancer.	Raloxifene and goserelin
	Phase 1 trial to study the effectiveness of perillyl alcohol in preventing the recurrence of breast cancer in women who have been treated with surgery with or without adjuvant therapy.	Perillyl alcohol
	Randomized phase 2 trial to evaluate the effectiveness of exemestane and raloxifene in treating postmenopausal women who have a history of ductal carcinoma in situ, stage I, stage II, or stage III breast cancer.	Exemestane and raloxifene
Cervical	Randomized phase 2 trial to study the effectiveness of LY353381 in preventing breast cancer in women who have hyperplasia.	LY353381
	Phase 1 trial to study the effectiveness of photodynamic therapy with lutetium texaphyrin in treating patients who have cervical intraepithelial neoplasia.	Lutetium texaphyrin
	Randomized phase 2 trial to study the effectiveness of applying topical imiquimod before abnormal cervical cells are removed in preventing cervical cancer in patients who have recurrent or persistent cervical neoplasia.	Imiquimod

*Continued on next page.*

**Table 2 (Continued)**  
**NCI-Sponsored Primary and Secondary Prevention of Cancer Studies**

Tumor type	Study title	Agent
Colon	Phase 1 trial to determine the dose amount of curcumin that can be tolerated to help in preventing colon cancer in healthy men and women.	Curcumin
	Randomized phase 2 trial to compare the effectiveness of celecoxib with or with eflornithine in preventing colorectal cancer in patients who have familial adenomatous polyposis	Celecoxib and eflornithine
	Randomized, double-blinded, phase 2 trial to determine the effectiveness of eflornithine plus sulindac compared to a placebo in preventing colorectal cancer in patients who have had surgery to remove benign colorectal polyps.	Eflornithine and sulindac
	Randomized clinical trial to determine the effectiveness of aspirin and/or folic acid in preventing the recurrence of colorectal polyps.	Aspirin and folic acid
	Randomized phase 2 trial to study the effectiveness of celecoxib in preventing colorectal cancer in patients who have a history of rectal polyps or colorectal neoplasia.	Celecoxib
Endometrial	Chemoprevention with Folic Acid	Folic Acid
	Randomized phase 2/3 trial to determine the effectiveness of exisulind in preventing the development and growth of polyps in patients who have familial adenomatous polyposis.	Exisulind
	Randomized phase 2 trial to compare different hormone therapy regimens in preventing endometrial cancer in women who have a genetic risk for endometrial cancer.	Hormone
	Phase 2 trial to compare the effectiveness of surgery alone with that of medroxyprogesterone followed by surgery in preventing endometrial cancer in patients who have endometrial hyperplasia.	Medroxyprogesterone
	Randomized phase 3 trial to study the effectiveness of medroxyprogesterone in preventing endometrial disorder in postmenopausal women who have ductal carcinoma in situ, lobular carcinoma in situ, Paget's disease of the nipple, stage I breast cancer, or stage II breast cancer and who are taking tamoxifen.	Medroxyprogesterone
Esophageal	Randomized phase 2 trial to study the effectiveness of celecoxib in preventing cancer in patients who have Barrett's esophagus.	Celecoxib
Lung	Randomized phase 1 trial to study the effectiveness of oltipraz in preventing lung cancer in people who smoke.	Oltipraz
	Randomized double-blinded phase 2 trial to study the effectiveness of isotretinoin with or without vitamin E for chemoprevention of cancer in persons at high risk of developing lung cancer.	Isotretinoin
	Randomized phase 3 trial to determine the effectiveness of selenium in preventing the development of second primary lung tumors in patients who have undergone surgery to remove stage I non-small cell lung cancer.	Selenium
	Phase 2 trial to study the effectiveness of celecoxib in preventing non-small cell lung cancer in tobacco smokers	Lung
	Randomized phase 3 trial to determine the effectiveness of a program to quit smoking with or without bupropion in treating patients who have undergone surgery for stage I or stage II non-small cell lung cancer.	Bupropion

**Table 2 (Continued)**  
**NCI-Sponsored Primary and Secondary Prevention of Cancer Studies**

Tumor type	Study title	Agent
Multiple myeloma	Randomized phase 2 trial to compare the effectiveness of dehydroepiandrosterone with that of clarithromycin in treating patients who may be at a high risk of developing multiple myeloma.	Dehydroepiandrosterone
Ovarian	Randomized clinical trial to compare the effectiveness of fenretinide followed by surgery with that of surgery alone in preventing ovarian cancer in patients who are at increased risk.	Fenretinide
Prostate	Randomized clinical trial to study the effectiveness of isoflavones in preventing prostate cancer	Isoflavones
	Randomized phase 2 trial to compare the effectiveness of a low-fat, high-fiber diet to that of a standard diet in treating patients who have prostate cancer.	Low-fat, high-fiber
	Randomized clinical trial to study the effectiveness of selenium in preventing prostate cancer in patients who have neoplasia of the prostate.	Selenium
	Randomized phase 2 trial to study the effectiveness of giving doxercalciferol before surgery in treating patients who have localized prostate cancer.	Doxercalciferol
	Randomized clinical trial to compare the effectiveness of isoflavones with that of lycopene before surgery in treating patients who have stage I or stage II prostate cancer.	Isoflavones or lycopene
	Randomized phase 2 trial to determine the effectiveness of efloornithine in preventing prostate cancer in men who are at high risk of developing the disease.	Efloornithine
	Randomized phase 2 trial to determine the effectiveness of efloornithine in preventing prostate cancer in men who are at high risk of developing the disease.	Efloornithine
	Randomized phase 2 trial to determine the effectiveness of soy protein supplement in preventing prostate cancer in patients who have elevated PSA levels.	Soy protein
	A chemoprevention study of an investigational drug in men with high grade prostate intraepithelial neoplasia (PIN).	Acapodene
	Randomized phase 3 trial to determine the effectiveness of selenium and vitamin E, either alone or together, in preventing prostate cancer.	Selenium and Vitamin E
Skin	Phase 1 trial to study the effectiveness of lycopene in preventing prostate cancer.	Lycopene
	Randomized clinical trial to study the effectiveness of acitretin in preventing skin cancers in patients with at least two previously treated skin cancers who have undergone organ transplantation.	Acitretin
	Randomized phase 2 trial to study the effectiveness of celecoxib in preventing skin cancer in participants exposed to ultraviolet light.	Celecoxib
	Randomized phase 2/3 trial to determine the effectiveness of celecoxib in preventing skin cancer in patients who have actinic keratoses.	Celecoxib
	Randomized phase 2 trial to determine the effectiveness of celecoxib in preventing basal cell carcinoma in patients who have basal cell nevus syndrome.	Celecoxib

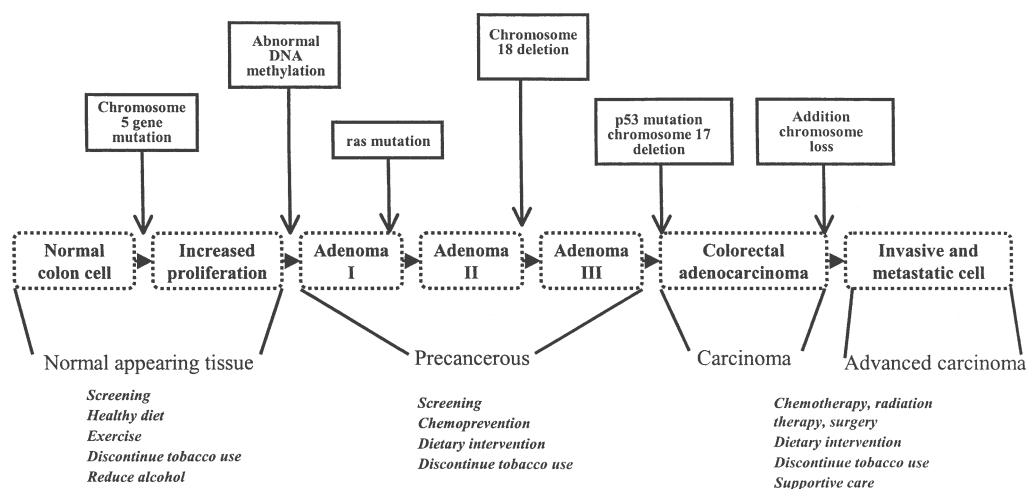


Fig. 1. Genetic alterations in colon carcinogenesis adapted from Vogelstein and colleagues, 1990. Initial genetic alterations cause an increase in proliferative capacity and probability for additional mutations. Subsequent genetic changes include point mutations in the oncogene *ras* and the tumor suppressor gene *p53*, and chromosome deletions and alterations in DNA methylation. (Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell*. 1990; 61:759–767).

## The Multistep Process of Carcinogenesis

In the late 1980s, Vogelstein and colleagues analyzed genetic abnormalities in colon cancer and proposed an etiologic combination of events for colon carcinogenesis (Fig. 1) (1). The model proposed that a series of mutations occur in the progression from normal cells to colorectal cancer and that these mutations are associated with the histologic features of the tumors. An initial mutation in chromosome 5p increases proliferative capacity. Subsequent genetic alterations include abnormal DNA methylation, point mutations in oncogenes and tumor suppressor genes (TSGs), and deletions of chromosome 18 (2–5). In this model, multiple genetic mutations must be accumulated; however, the order in which they are accumulated can vary. It has been hypothesized that all cancers develop similarly in that many mutations must occur, providing many points at which interventions interfering with carcinogenesis may be successful. TSGs, oncogenes, and regulatory proteins show promise as targets for treatments of carcinogenesis at various stages and have potential for serving as markers for early diagnosis.

In the early 1980s, Moolgavkar and Knudson proposed a model of carcinogenesis hypothesizing that more than one mutation is required for conversion of a normal somatic cell into a malignant cell (6). This model takes into account several properties of cell self-renewal. First, immature progenitor cells comprise a relatively small proportion of any given tissue or organ. Second, because all proliferating progenitors have a limited life-span, mutations acquired by any one cell will disappear after a given number of cell divisions. Third, proliferative rates differ in most organs and tissues during the lifetime of an animal. In this model, transformation from a normal to a malignant phenotype requires only two mutations. Cells with one mutation would still differentiate; however, after a second mutation, self-renewal is greatly increased and differentiation becomes abnormal.

## Signal Transduction and Treatment of Carcinogenesis

The process of carcinogenesis is regulated by a multitude of molecular mechanisms. A prerequisite for understanding the mechanisms of multistep carcinogenesis is the determination of which parts of the genome are structurally altered and the specific roles of the proteins of each altered gene. Dysregulation of tumor suppressor proteins and oncogene products is often the result of mutations harbored within the promoter region or coding sequence of the genes themselves. These genes can be expressed inappropriately through abnormal activity of the proteins by which they are directly regulated, including enzymes responsible for their activation, and transcription factors that regulate their expression by binding to specific sequences called *cis* elements, within the promoter region of the respective genes.

### Tumor-Suppressor Genes

#### *p53*

The p53 protein is a TSG expressed in most cells. Wild-type p53 protein functions to regulate the processes of the cell cycle, apoptosis, differentiation, DNA replication, and DNA repair. The p53 protein forms tetrameric structures in the cytosol, then translocates to the nucleus, where the complex binds directly to DNA within a promoter region and elicits transcriptional activation of target genes. Mutations in the *p53* gene have been found in almost all tumor types, including carcinomas of the colon, breast, lung, esophagus, stomach, liver, and bladder. It is estimated that 50% of all human tumors contain a mutation(s) within the *p53* gene, resulting in expression of dysfunctional p53 protein (7–10). Mutated p53 proteins are unable to bind DNA and/or other proteins and therefore cannot exert control of the cell cycle and apoptosis in precancerous cells (11). Ultimately, proliferation becomes dysregulated in a small portion of cells containing mutated p53.

#### *β-Catenin*

β-Catenin is a transcription factor that is strongly linked to colorectal and other forms of cancer. The protein, adenomatous polyposis coli (APC), which is regulated by TSGs, is normally expressed at low levels in the cytosol. Genetic mutations in APC have been clearly linked to familial polyposis and a greatly increased risk of colorectal cancer. Additionally, 70–80% of all colorectal tumors exhibit mutations in APC (12–14).

In a normal cell, APC binds to β-catenin in the nucleus. This initiates a cascade of molecular events that results in degradation of β-catenin. Mutated APC is unable to bind to β-catenin, causing accumulation of cytosolic β-catenin. Ultimately, β-catenin is translocated to the nucleus, where it binds with transcription factors in the TCF/LEF family. This β-catenin–TCF/LEF complex binds DNA and upregulates oncogenes such as *c-myc*; cyclin D1, which is involved in cell-cycle regulation; and matrix metalloproteinase-7 (MMP-7), which is involved in degradation of the extracellular matrix (ECM) (15–17). The increase in transcription of these genes through the APC/β-catenin/TCF/LEF pathway has been directly implicated in carcinogenesis.

### Oncogenes

#### *Her-2/neu*

Her-2/neu is a cell-surface receptor that is overexpressed in many breast, gastrointestinal (GI), lung, and genitourinary cancers (18). Her-2/neu is thought to function as a growth factor receptor and has a role in cell differentiation, adhesion, and motility.

The *Her-2/neu* gene, a tyrosine kinase family member, encodes a transmembrane receptor-like protein that is structurally similar to the epidermal growth factor receptor (EGFR). Under normal conditions, molecules such as EGF or heregulin bind to and activate *Her-2/neu*. Subsequently, *Her-2/neu* heterodimerizes with other members of the EGFR family, which leads to signaling events that regulate growth, differentiation, and alterations of cell adhesion properties (18). During carcinogenesis, when *Her-2/neu* levels are aberrantly increased, the protein can be activated in the absence of cell-surface signals (19). The amplification of *Her-2/neu* is thought to contribute to a neoplastic phenotype through its ability to induce resistance to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). The constitutive increase in *Her-2/neu* activity upregulates certain cyclin D family proteins and dysregulates the G1/S-phase cell-cycle checkpoint.

In cancer, the *Her-2/neu* gene is both amplified and overexpressed. Gene amplification of *Her-2/neu* has been reported in 20–30% of all primary breast and ovarian tumors. Furthermore, *Her-2/neu* is overexpressed in 90% of all cases of ductal carcinoma *in situ* (DCIS) (19). Several lines of evidence suggest that *Her-2/neu* overexpression directly contributes to the pathogenesis and aggressiveness of many tumor types.

### *Myc*

Amplification of *c-myc* is associated with many tumor types including ovarian (20,21), breast (22), and small cell lung cancers (SCLC) (23,24). Translocations of the *c-myc* gene occur in approx 80% of all Burkitt's lymphomas (25), while family member *n-myc* is amplified in 25% of all neuroblastomas (26).

The *c-myc* gene is a member of the helix-loop-helix basic leucine zipper superfamily. On heterodimerization with the appropriate subunit, *c-myc* protein binds to *cis* elements within the promoter region of target genes and elicits transactivation activity (27). These *c-myc*-controlled genes modulate many cellular mechanisms, including cell-cycle control, differentiation, adhesion, and apoptosis. Both translocation and amplification result in greatly increased numbers of *myc* genes. Ultimately, overexpression of *myc* is directly linked to the pathogenicity and aggressiveness of many human tumors.

## **Transcription Factors**

### *Activator Protein-1*

Activator protein-1 (AP-1) was first observed to be expressed in response to the tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and is thought to have a direct role in malignant transformation. Two of its frequent components, *c-Jun* and *c-Fos*, are encoded by protooncogenes (28). AP-1 has been shown to influence the invasiveness of tumor cells. Genes controlled by AP-1 are overexpressed in almost all tumor cancer, and modulate tumorigenesis by transactivating genes that regulate invasion, motility, angiogenesis, and apoptosis (29).

The AP-1 transcription factor complex is either a homodimer or heterodimer composed of proteins belonging to the Jun, Fos, Jun-dimerizing partners, and activating transcription factors families. Dimerization of these proteins allows the complex to bind DNA and induce transcription of target genes. AP-1 activity is modulated by the differential expression of AP-1 proteins in response to stimuli. Overexpression of various AP-1 family members at the transcriptional level may have a primary role in many human cancers (29–33).

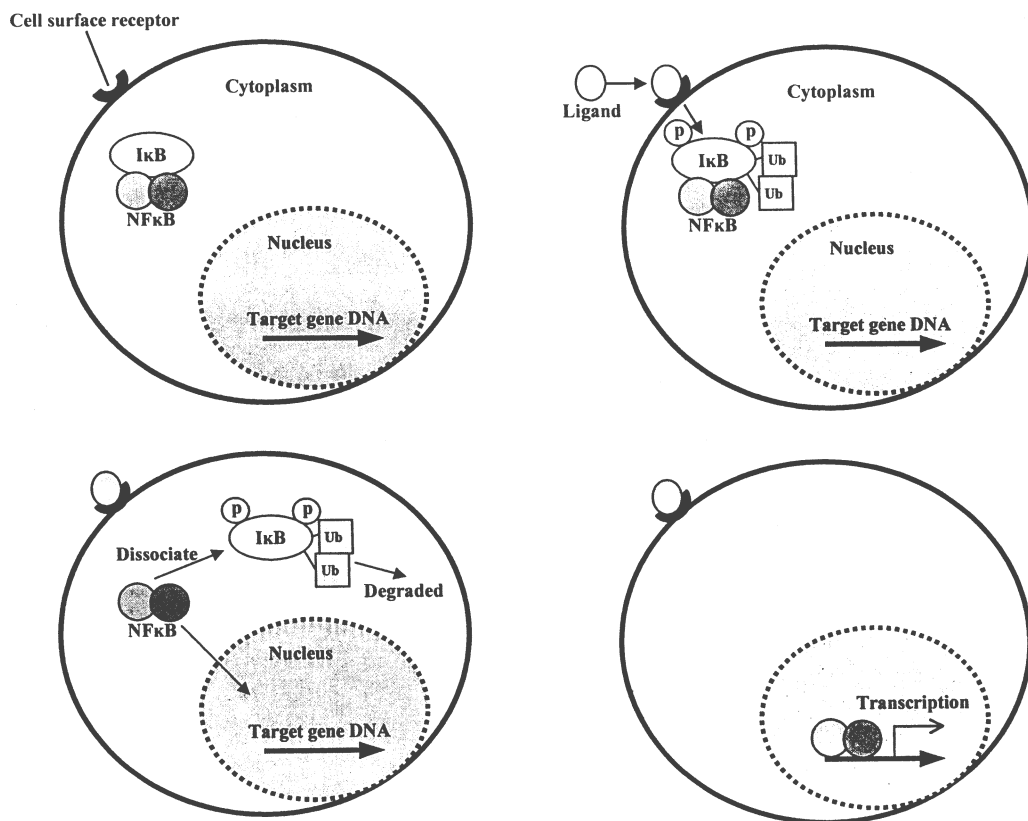
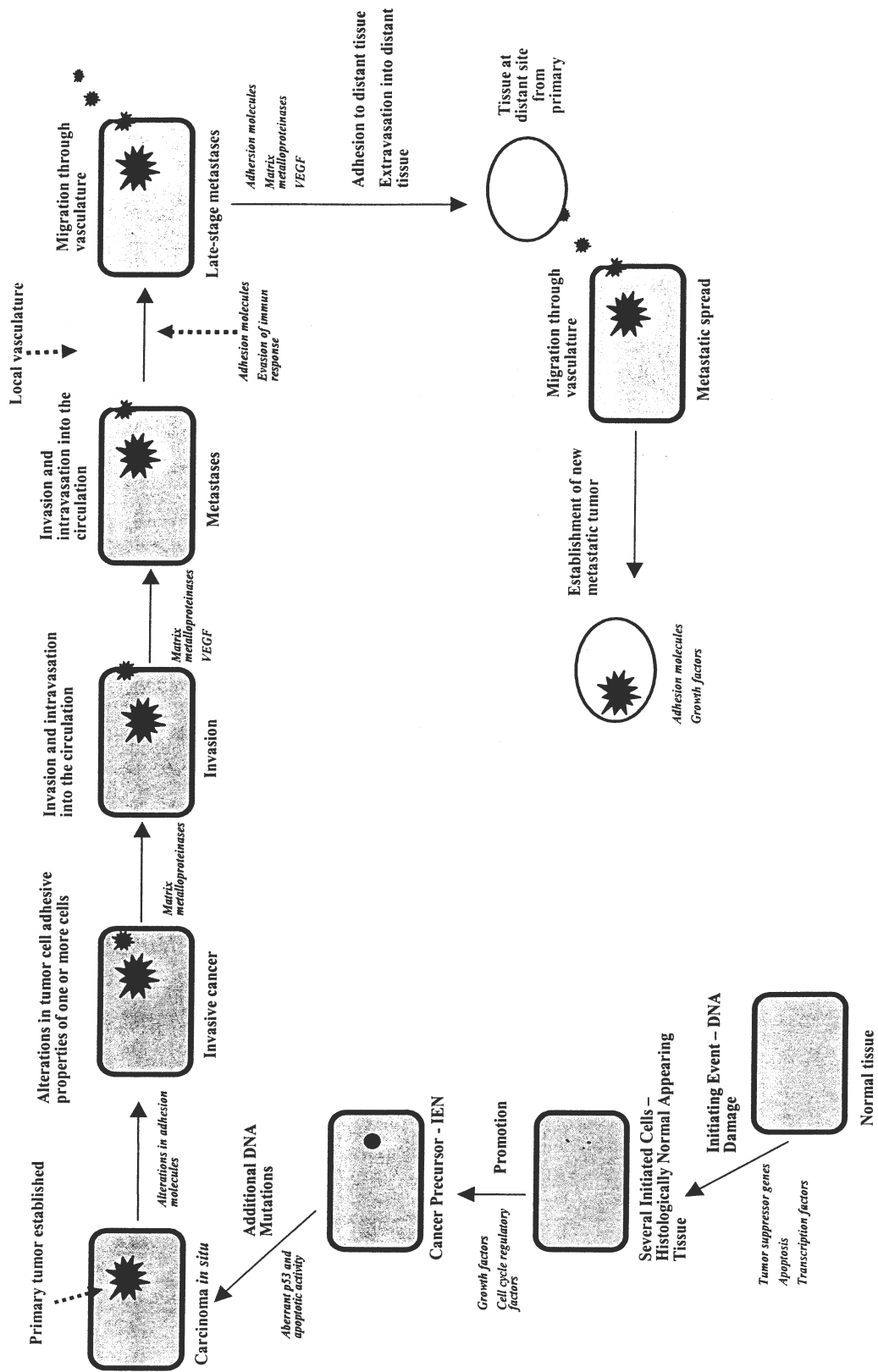


Fig. 2. Regulation of NF- $\kappa$ B. I $\kappa$ B is phosphorylated in response to ligand binding the respective cell-surface receptor. The I $\kappa$ B subunit is rapidly phosphorylated and ubiquitinated and dissociates from NF- $\kappa$ B before undergoing degradation. The NF- $\kappa$ B heterodimer then translocates to the nucleus, where it binds DNA and regulates transcription of target genes.

### Nuclear Factor- $\kappa$ B

Modulation of expression and/or activity of TSGs, oncogenes, and transcription factors have shown promise for mechanisms of chemoprevention. Furthermore, expression of mRNA and proteins can serve as surrogate end point biomarkers to evaluate efficacy of a chemopreventive agent. Because expression of these proteins is regulated by more than one mechanism, intervention is possible at several points. For example, the inactive form of NF- $\kappa$ B, which is known to regulate genes involved in apoptosis, cell-cycle arrest, and inflammation, is sequestered in the nucleus by its inhibitory subunit, I $\kappa$ B. To become activated a cascade of events must occur. In response to a nuclear factor- $\kappa$ B (NF- $\kappa$ B) regulatory ligand binding to its respective cell-surface receptor, the I $\kappa$ B subunit is phosphorylated at two Ser residues and is subsequently ubiquitinated and degraded by the proteasome after dissociation from the NF- $\kappa$ B heterodimer. This dissociation unmasks the nuclear localization sequence on the NF- $\kappa$ B heterodimer, which translocates to the nucleus, where it can then bind DNA and elicit transactivation of target genes (Fig. 2) (34–38). This complex mecha-





nism of activation provides several points at which NF- $\kappa$ B transactivation activity can be inhibited. Some agents, including inhibitors of cyclooxygenase (COX) enzymes (39), have been shown to block phosphorylation of I $\kappa$ B causing inhibition of NF- $\kappa$ B activation while other agents, such as a p50-binding peptide, block the nuclear localization sequence of the active NF- $\kappa$ B heterodimer. This mechanism allows dissociation of NF- $\kappa$ B from the inhibitory subunit but blocks nuclear translocation and, therefore, NF- $\kappa$ B transactivation activity.

Elucidation of the molecular mechanisms of activity of potential chemopreventive agents has revealed that regulation of TSGs and oncogenes may provide insight into successful treatment of carcinogenesis. Because transcription factors directly regulate gene expression, agents that can mediate transcription factor activity could serve as potential chemopreventive agents.

## Matrix Metalloproteinases

### MMPs

In most malignancies for which solid tumors are characteristic, morbidity and mortality are caused primarily by metastatic spread of the primary tumor(s) to distal sites within target organs defined by tumor type. The process by which metastases develop comprises a complex cascade of steps that are regulated by a multitude of proteins (Fig. 3). Once a primary tumor is established, alterations in cell-adhesive properties, which can be regulated by integrins and cadherins, permit one or more cells to detach from the primary tumor cells and locally surrounding ECM matrix (40–44). Subsequently, the cells must intravasate into local vasculature. Cells must then be transported through the vascular or lymphatic system and will form metastatic tumors successfully only if the host immune system is evaded (45–49). At a site distant from the primary tumor from which they were derived, tumor cells extravasate into the surrounding parenchyma and organs, where new growth can be established.

One of the key steps to metastatic invasion includes intravasation of primary tumor cells into local vasculature and extravasation of the cells into surrounding tissue distant from the primary lesion. These processes are mediated, in part, by MMPs, a family of enzymes that require zinc for catalytic activity and are responsible for degradation of the ECM (50). Nonspecific MMP inhibitors are often designed to block the zinc-binding region, with which all MMP family members share identity (51). More than 27 MMPs have been identified. Subclasses have been formed according to substrate specificity; however, each MMP has multiple substrates which it is capable of degrading. More recently, a membrane-bound subclass has been identified (52,53).

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Fig. 3. (*Opposite page*) Steps to development of metastatic cancer. Tumor cells detach from a primary tumor, invade through the basement membrane of the tissue in which the tumor is established, and then intravasate into the circulatory system. At a distant site, the cells extravasate out of the vasculature and into tissue where a metastatic tumor can be established. Adhesion properties of cells are regulated by integrins, which mediate cell-matrix interaction, and cadherins, which mediate cell-cell adhesion. Degradation of the ECM and basement membrane is regulated by MMPs and TIMPs. Establishment of a metastatic lesion also requires the activity of dysregulated cytokines and growth factors.

### ***MMPs and Carcinogenesis***

The progression of solid tumors is modulated not only by a net increase in cell proliferation, but also by inhibition of sensitivity to signals that normally result in apoptosis. Furthermore, growth of solid tumors is also mediated through up regulation of growth factors, including EGF (54,55), transforming growth factor- $\beta$  (TGF- $\beta$ ) (56,57), and insulin-like growth factor (IGF) (58–60), which directly regulate MMP expression (61–64).

Regulation of MMP is complex. Expression of some MMP family members is upregulated during various stages of embryonic development such as heart valve formation (65), as well as in response to tissue injury (66) or other stresses that require remodeling of the ECM. MMPs are secreted as inactive zymogens that require the removal of the amino-terminal prodomain to achieve catalytic activity. Once active, most MMPs can be inactivated by the tissue inhibitors of MMPs (TIMPs), which specifically bind the hemopexin-binding domain present in most MMPs. All secreted MMPs characterized to date share in common three structural domains including a leader sequence, which is required for secretion of the inactive zymogen, a prodomain that inhibits catalytic activity of the uncleaved enzyme, and the catalytic domain that contains the zinc-binding region.

It has been shown both in vitro and in vivo that invasion of a primary tumor and metastatic processes are facilitated, in part, by MMP (62,67–69). Thant et al. (70) showed that Ras-mediated signaling has a critical role in activation of MMP-2 and, subsequently, in the invasiveness of src-transformed cells in vivo using a Boyden chamber assay. It has been shown, in a melanoma cell line, that stable transfection with dominant negative type I collagenase (MMP-1) inhibited the invasive capacity of the cells through Matrigel. Inhibition of MMP-1 activity was confirmed by zymography (70). Bu et al. (71) examined MMP-2 expression in hepatocellular carcinoma. They demonstrated a correlation between the amount of MMP-2 present in tumor tissues and surrounding parenchyma and the level of invasion of the cancer. Tissue samples were examined using immunohistochemistry (IHC) analyses (71). Overexpression of MMP has been observed in human tumors of the colon, breast, skin, and head and neck.

### ***MMPs as Targets for Carcinogenesis***

MMP inhibitors have been developed and are currently in phase 1 and 2 clinical trials in many tumor types. Marimistat, a broad-spectrum MMP inhibitor, is being tested in the clinic in breast cancer (72) and pancreatic cancer (73,74). Phase 2 studies, which have used marimistat alone or in combination with other cytotoxic agents, have produced encouraging results with improved survival. Pivotal phase 3 trials are under way for the use of marimastat in advanced pancreatic cancer and as an adjuvant therapy in patients after resection of pancreatic cancer (75,76). Another MMP inhibitor, batimistat, is also in clinical trials for treatment of pleural effusion caused by increased expression of MMP by stromal cells in the lung of patients with cancer (77).

Because MMPs participate in several stages of cancer progression, they may provide a valuable marker for diagnosis and prognosis, and they may also provide therapeutic targets for prevention of cancer progression. One aspect of physiologic homeostasis is maintained by preservation of balanced expression between MMP and TIMP. MMP-2 is significantly overexpressed in tissue isolated from early stage human colon tumors; and the ratio of mRNA of MMP-2 to TIMP-2 is twofold lower in colon

cancer tissue compared with normal colon tissue (78,79). The ratio of MT1-MMP to TIMP-2 is also significantly reduced. These data suggest that MMP-2 may have a role in malignant transformation of the colon.

It is of great interest that natural products being studied for their chemopreventive properties have been shown to inhibit expression of MMP regulatory proteins. Genistein, an isoflavone found in soy products, which has demonstrated antiangiogenic properties in preclinical testing, has been shown to inhibit the invasive capacity of two human breast cancer cell lines, MCF-7 and MDA-MB-231. In addition to causing cell-cycle arrest in G2- to M-phase and apoptosis, genistein inhibits expression of MMP-9 and upregulates expression of TIMP-1, which is known to block MMP-9 activity (80, 81). Genistein has also been shown to have activity against cell lines established from human prostate carcinomas (80,81), ovarian cancers (80,81), and nonmelanoma skin cancers (82,83). Green tea catechins, including epigallocatechingallate, also inhibit MMPs.

Preliminary studies examining vitamin D suggest that potential chemopreventive effects of vitamin D may be mediated, in part, by regulation of MMP expression and activity (84–86). In an in vitro invasion assay, calcitriol, the hormonal form of vitamin D, inhibits the invasive capacity of the human prostate cancer cell line, DU-145. Furthermore, IHC analyses have shown colocalization of vitamin D receptors and MMP-1, -3, and -9 in cultured human articular chondrocytes.

## **Treatment of Carcinogenesis in Specific Tumor Types**

### ***Opportunities for Treatment of Cancer Precursors: IEN***

Development of chemopreventive agents is based on use of validated intermediate biomarkers that directly correlate with cancer incidence and are feasible for cohort studies. Because chemopreventive agents are applied over a long period of time and the population of study is relatively healthy, the agents must have an acceptable toxicity profile. Because of its slow development, cancer is not a feasible end point for most chemoprevention studies. Therefore, intermediate end points are crucial. Of particular value are changes in nuclear chromatin pattern and early stage changes that define IEN (87–89). These early neoplastic changes show a causal relationship to cancer. They may serve as target lesions in clinical studies and as standards against which other biomarkers can be compared and validated. IEN can provide a target in many tumor types including lung (90), breast (91), colon (92), prostate, skin, bladder, and cervix (90). Cohorts for these and other tumor types have been identified for phase 2 clinical trials evaluating the effects of chemopreventive agents on IEN. For example, patients with adenomatous polyps serve as a cohort for colon cancer. Precursor lesions provide an opportunity for intervention in the earliest stages of carcinogenesis when prognosis is most likely favorable.

### ***Prostate Cancer***

Prostate cancer is the second most frequently diagnosed malignancy in men, surpassed in incidence only by nonmelanoma skin cancers. In 2001, approx 198,000 men were diagnosed with prostate cancer and an estimated 31,000 men died (93). Although significant progress has been made in the past decade that has improved our understanding of the disease, much remains to be learned about the causes, early detection markers for diagnosis and determination of prognosis, treatment, and prevention. A large part of the challenge for treatment of prostate cancer is the inability to differenti-

ate between primary tumors that will result in fatal disease from a tumor that will grow very slowly and hence be clinically insignificant in some men. To help overcome this challenge, several areas of research must be approached. First, it is critical to identify genetic, physiologic, and environmental factors that contribute to increased risk. Second, molecular and cellular processes contributing to development, invasion, and metastases of prostate cancer must be examined for development of improved early detection methods and targeted therapies. Third, continuous epidemiologic studies must continue to understand the relationship of incidence and mortality in different populations and within families. Finally, *in vitro* and *in vivo* models of prostate cancer must be developed to facilitate preclinical studies that will lead to therapeutic and chemopreventive agents.

Research within the past decade has begun to characterize the molecular and cellular processes involved in development and progression of prostate cancer. For example, significant strides in research have allowed better understanding of the processes of initiation, cell growth, and invasion. In addition, influences of the interactions between cancer cells and their environment, which contribute to metastatic mechanisms, are the subject of intense study. Some of these factors include alterations in integrin and cadherin expression on the surface of cancer cells (94–96), MMP expression (97–99) that contributes to degradation of the ECM during invasion and metastases, hormone-independent growth of prostate cancer cells that have become refractory to androgen ablation therapy (100,101), and changes in the normal patterns of apoptosis (102).

### *Prostatic IEN*

Prostate cancers are characteristically heterogeneous and multifocal in nature and can have diverse clinical and morphologic manifestations (103). Although the molecular basis for this heterogeneity has yet to be elucidated, further study of prostatic intraepithelial neoplasia (PIN), a pathologic diagnosis considered to be a precursor lesion for prostate cancer, may help contribute to characterization of specific cancers. Prostate cancer may arise from other types of precursors; however, PIN is considered to be the most likely precursor for prostate cancer at present. The strong genetic similarities between PIN and carcinoma of the prostate suggest that evolution and clonal expansion of PIN leads to development of cancer (104–107). Data examining age and race as risk factors have revealed that black men develop more extensive high-grade PIN at a younger age than white men, and some studies have shown that men with PIN will be diagnosed with prostate cancer within 10 yr of their original biopsy (108,109). It is of interest that pronounced genetic heterogeneity is characteristic of both PIN and carcinoma. Furthermore, multiple foci of PIN and carcinoma can arise independently within the same prostate, suggesting a field effect of factors that influence carcinogenesis.

High-grade PIN lesions and prostatic tumors share a broad spectrum of molecular and genetic abnormalities, including loss within the chromosome regions 8p, 10q, 16q, and 18q, and gain within chromosome regions 7q31 and 8q (110,111). Other abnormalities that have been identified in both high-grade PIN lesions and prostatic carcinoma tumors include amplification of the oncogene *c-myc* (112), aberrations in nuclear chromatic pattern (88), and altered activity of telomerase (113,114), cell-cycle regulators (115), proliferative indices (116,117), and markers of apoptosis (118–120). These data suggest that high-grade PIN is an intermediate stage between benign prostatic epithelium and prostatic carcinoma, and it may be critical in early stages of carcino-

genesis and neoplastic progression in the prostate. High-grade PIN could therefore potentially serve as an ideal target for early diagnosis of prostate cancer and development of agents to prevent progression of early stages of carcinogenesis.

In studying high-grade PIN as a precursor to prostate cancer, it is important to remember that endocrine therapy causes alterations in morphology of PIN, making it more closely resemble normal prostatic epithelium (105,108). Endocrine therapy-induced changes in molecular markers of PIN and induction of resistance to endocrine therapy are important areas of study.

### *Etiology and Prevention*

Numerous epidemiologic studies have documented that increasing age, family history, and race are contributing factors to prostate cancer (121-123). These studies have shown that the likelihood of developing prostate cancer is greater among men with a family history, suggesting that genetic susceptibility may increase risk. Prostate cancer incidence and mortality rates have been shown to vary among countries and racial ethnic groups, with black men having the highest incidence and mortality in the world and Asian men having the lowest (93). The major goals in the areas of etiology and prevention of prostate cancer include identifying genetic, biochemical, environmental, and lifestyle factors, and their interactions that define prostate cancer risk, play direct roles in early and advanced stages of carcinogenesis, and lead to the development of novel strategies for prevention and early detection.

### SELENIUM AND VITAMIN E

Epidemiologic studies suggest an inverse relationship between intake of dietary selenium and incidence of cancer (124-126). Numerous animal studies have shown that dietary supplementation with selenium reduces cancer incidence in a variety of animal models, including melanoma and cancers of the colon, breast, liver, esophagus, head and neck, pancreas, kidney, and lung (127-129). One of the central findings of the Nutritional Prevention of Cancer study conducted at the University of Arizona was a >60% reduction in the incidence of prostate cancer in participants randomized to 200 µg per day of selenium compared with a placebo group (130-133). These findings led to the development of additional, randomized, blinded, placebo-controlled, clinical studies testing the effects of selenium on prevention of primary and secondary prostate cancer.

The Selenium and Vitamin E Cancer Prevention Trial (SELECT) is a National Cancer Institute (NCI)-sponsored randomized, prospective, double-blind study designed to determine whether selenium and/or vitamin E decrease the risk of prostate cancer in healthy men. SELECT is coordinated by the Southwest Oncology Group and plans to enroll a total of 32,400 healthy men at >400 clinical study sites in the United States, Puerto Rico, and Canada (134). Preclinical, epidemiologic, and phase 3 data suggest that selenium and vitamin E have potential efficacy for prostate cancer prevention. The four cohorts of this study are selenium/vitamin E, selenium/placebo, placebo/vitamin E, and placebo/placebo. Enrollment began in July 2001 and the trial is expected to be completed in 2013.

The Arizona Cancer Center at the University of Arizona is conducting three additional clinical studies examining the effect of selenium on primary and secondary prevention of prostate cancer in several populations (135). The Negative Biopsy Study is an NCI-sponsored study in which 700 men, who have had at least one negative prostate

biopsy within 1 yr of study enrollment, are randomized to receive either 200 or 400  $\mu\text{g}/\text{d}$  of selenium vs placebo. Endpoints include prostate-specific antigen (PSA) velocity, development of biopsy-proven prostate cancer, and serum markers including alkaline phosphatase and chromogranin A. The Watchful Waiting study, which is also NCI sponsored, is randomizing 220 men with biopsy-proven prostate cancer who have elected not to undergo surgery, radiation, hormone therapy, or any other type of therapy, and are  $<85$  yrs of age. Treatment groups include 200 or 800  $\mu\text{g}$  of selenium/d or placebo. Endpoints will include PSA velocity, time to progression, time to treatment, and alkaline phosphatase and chromogranin A levels. Statistical analyses for this study will be stratified by Gleason score. The Preprostatectomy Study is sponsored by the Department of Defense. This study is enrolling men who have been recently diagnosed with prostate cancer and are scheduled for a radical prostatectomy between 3 and 6 wks from the time of enrollment. During that time, they will be randomized to receive 200 or 400  $\mu\text{g}$  of selenium/d or placebo. In this study, amounts of selenium in prostate tissue will be measured from the time of the original diagnostic biopsy and from the radical prostatectomy to determine whether selenium taken orally can affect selenium concentrations in prostate tissue. Tissue will be analyzed for markers of cell growth and apoptosis. By the end of 2002, the Negative Biopsy Study accrued  $>50\%$  of the patients planned. The Watchful Waiting and Preprostatectomy studies accrued approx 75% of the patients planned.

#### ANDROGEN INHIBITORS

Research has suggested that androgenic stimulation over time increases risk of prostate cancer (136–138). The corollary to this hypothesis is that a decrease in long-term androgenic stimulation may lower the chances of developing prostate cancer. Decreasing androgenic stimulation of the prostate with 5- $\alpha$ -reductase inhibitors, such as finasteride, has been shown to decrease prostate volume and may prevent prostate cancer (139–141). A large, long-term clinical trial has completed enrollment of  $>15,000$  participants and is completing follow-up data to determine whether finasteride can prevent prostate cancer (142). Results are expected in 2004.

#### DIFFERENTIATION AND ANTIPROLIFERATIVE AGENTS

Other classes of compounds that show promise in the prevention of prostate cancer progression include differentiation and antiproliferative agents, some of which have been used to successfully treat certain types of leukemias (143–145). Research suggests that these agents, including natural products such as green tea components and isolates from lemon peel, may induce terminal differentiation (arrest in G0), induce differentiation to a mature cell with cellular functions and a growth pattern similar to nonmalignant cells, or trigger apoptosis (146–148). A potential advantage of differentiating agents is that they offer a reduced toxicity profile compared with other cancer therapies. These agents may provide an alternative therapy for men afflicted with advanced prostate cancer or they may show promise as low-toxicity agents administered chronically to men at high risk of developing prostate cancer. Clinical trials are needed to define the role of these agents in primary and secondary prevention.

#### GLUTATHIONE-S-TRANSFERASE 1 REGULATION

Environmental factors, including diet, appear to play a role in the risk of developing prostate cancer. Many candidate dietary components have been proposed to influence human prostatic carcinogenesis, including fat, fruit, and vegetable components; antioxidants, and various micronutrients (149–152). The specific roles that dietary agents play

in promoting or preventing carcinogenesis have not been elucidated. Evidence suggests that *GSTP1*, the gene encoding the pi-class glutathione-S-transferase (GST), may be critical in the maintenance of homeostasis in normal prostatic epithelial cells (153,154). Although *GSTP1* can be detected in normal prostatic epithelium, functional GSTP1 polypeptides have been shown to be absent in prostatic cancers. Loss of GSTP1 function also appears to be characteristic of PIN lesions. These data provide evidence that agents capable of restoring GSTP1 expression could prevent progression of PIN to cancer.

### *Chromosomal Alterations in Prostate Carcinogenesis*

Abnormalities in chromosomes 8 and 10 may be associated with development of prostate cancer. Preliminary findings using restriction fragment length polymorphism analyses showed consistent alterations in genetic information located in chromosomes 8 and 10 in men with prostate cancer (155). Subsequent studies of alterations in chromosomes 8 and 10 have been completed. NCI-sponsored studies suggested that a deletion in chromosome 8 (8p21) was present in 80% of prostate cancers and approx 63% of precancerous prostate lesions (156–158). Such a finding suggests that abnormalities in chromosome 8p21 may be associated with early development of prostate cancer, and changes in protein expression associated with this deletion may serve as early diagnostic markers or targets for treatment of early prostate carcinogenesis. *SOX7* gene, located on chromosome 8p22 is downregulated in primary prostate cancer and prostate carcinoma cell cultures (159,160). This gene is thought to be a TSG for prostate cancer and other solid tumors, including kidney and breast. Other identified genes that are potentially involved in the carcinogenesis of prostate cancer include *LZTS1*, located on chromosome 8p22 (161,162) and *KLF6* (163), located on chromosome 10p.

### *Treatment of Advanced Carcinogenesis in Prostate*

The process by which solid tumors metastasize to bone is multistep and is dependent on paracrine interactions between tumor cells and normal cells. Prostate cancer bone metastases, contrary to breast cancer bone metastases, which evoke hormone-mediated bone destruction, actually stimulate osteoclasts to produce new bone growth (164–166). Mechanisms identified as relevant to metastatic spread of prostate cancer to bone include several factors. One of the steps in formation of bone metastases requires the vascular spread of cancer cells to bone. This process is partially regulated by MMPs (167,168), a family of enzymes that degrade ECM, and growth factors, such as vascular endothelial growth factor (169,170). For tumor cells to successfully invade the osseous tissue, they must adhere to the bone microvasculature and ECM components. These processes are regulated by growth factors and adhesion molecules. Finally, the formation of bone metastases requires aberrant expression and activity of growth factors and chemoattractants in the area of metastases and tumor-induced local osteoblastic proliferation.

The ejaculate protein endothelin-1 (ET-1) from prostate cancer cells, is enhanced by bone contact, which blocks osteoclastic bone resorption. In the presence of bone tissue in coculture, androgen-independent prostate cancer cell lines expressed a high level of ET-1 mRNA and protein. Bone reabsorption was blocked in a dose-dependent manner relative to the number of prostate cancer cells present (171). Neutralizing antibodies (Abs) against ET-1 blocked the inhibition of bone reabsorption caused by the prostate cancer cells (172). These data suggest that ET-1 may be a valuable target for therapies to prevent progression of metastatic spread of prostate cancer to bone.

### **Nonmelanoma Skin Cancers**

Nonmelanoma skin cancer (NMSC) is the most common type of cancer, significantly impacting health care in developed nations. Estimates for the United States projected that skin cancers would comprise 46% of all diagnosed malignancies in 2002 (173,174). These estimates were probably lower than the number of cases, because unlike other cancers, many skin cancers are treated or removed in clinics without being reported to cancer registries. In spite of the tremendous number of NMSC cases, mortality of this disease is relatively low. Death rates are <1.5 per 100,000 (175). Morbidity can be dramatic owing to excision of lesions in cosmetically sensitive areas (176,177). While the mortality owing to skin cancer is relatively low compared with other cancers, the contribution of skin cancer to the total direct medical costs of cancer is not insignificant. For NMSC, the total annual cost of care from 1992 to 1995 was estimated to be US\$426 million in the Medicare population alone (178).

In 1775, the London surgeon Percivall Pott (179) reported the first skin cancer tumor model to appear in the literature. His report of scrotal cancer in chimney sweeps is considered to be the historical beginning of cancer research, as he delved beyond treatment into the etiology of the disease (180). His discovery of the linkage between scrotal carcinoma and soot exposure was the first evidence of a cancer cause and launched several hundred years of research leading to the discovery of chemical carcinogens and their mechanisms of action.

Almost all NMSCs are keratinocytic and originate in the epidermis. Approximately 80% are basal cell carcinoma (BCC) (181). These neoplasms, originally described in 1827 (182), appear to originate from basal cells of the epidermis and occasionally those of the infundibular and outer root sheath of the hair follicles (176). These slow-growing tumors are locally invasive and rarely metastasize; however, morbidity can be high, because these tumors are often disfiguring and located in facial areas. Squamous cell carcinoma (SCC), the other major form of NMSC, originates in the keratinizing cells of the epidermis. These tumors are generally more aggressive than BCC and have a much higher potential for metastasizing (183,184). Mortality from NMSC is mainly due to SCC.

Chronic exposure to ultraviolet (UV) radiation in sunlight (185) and fair skin that is susceptible to sunburns appear to be important risk factors in development of NMSC. Increasing frequency of exposure, age, immune status, male gender, and DNA repair disorders such as xeroderma pigmentosum contribute to increased risk (181), as does a history of BCC or SCC (186–188). Conversely, a history of cutaneous melanoma appears to increase the risk of NMSC (189). The association between UV radiation exposure and cancer is strong for SCC, but less well defined for BCCs—approx 30% of BCCs originate in anatomic sites receiving minimal sunlight exposure (176). Skin temperature may be a factor (190). Oxidation of cellular biomolecules due to UV radiation exposure is believed to comprise the primary pathway for both aging and carcinogenesis of skin. Continued increases in NMSC incidence rates can be expected over the next several decades as the population ages and larger amounts of UV radiation reach the earth's surface owing to depletion of the ozone layer (191).

Patients who have undergone solid-organ transplant procedures and are at extremely high risk of SCC are solid-organ transplant recipients. A comprehensive study of 5356 transplant recipients in Sweden showed that these patients experienced a 100-fold increase in relative risk of NMSC, almost exclusively in sun-exposed areas (192). The increased frequency of SCC in these patients, especially in individuals with chronic



actinic damage, is presumably due to long-term immunosuppressive therapy (193), although nonimmune mechanisms and direct effects of immunosuppressive drugs may play a role (194).

### *IEN as a Target for Prevention of Squamous Cell Carcinoma of the Skin*

Several precancerous dermatoses may occur before development of NMSC. Actinic keratosis ([AK], also known as solar or senile keratosis) is by far the most common precancerous dermatosis and is attributable to UV light (195). AK results from proliferation of transformed neoplastic keratinocytes confined to the epidermis. Clinically, AK appears as red, scaly, nonsubstantive papules on chronically sun-exposed areas such as the face, ears, and dorsal surfaces of forearms and hands (196). Histologically, AK is characterized by dysplasia of keratinocytes with loss of cellular polarity and nuclear atypia. The diagnosis of SCC is made when these atypical cells pass through the basement membrane, invading the underlying dermis. AK is cytologically indistinguishable from SCC (197).

AK is an extremely common lesion, especially in older white populations. In the United States, an estimated 3.7 million physician office visits per year for AK were made in 1993 and 1994 (198). In spite of these epidemic proportions of AK, few comprehensive epidemiologic data are available for AK incidence in North American populations. These data are difficult to collect due to variability in diagnosis and treatment, and the lack of reporting. Based on limited available data, the best estimate for current prevalence of AK ranges from 5 to 14% in the United States (199). In high-risk groups, prevalence may be as high as 26% (200). In Australia, where skin cancer incidence is highest, prevalence of AK ranges from 40 to 60% in the adult population (201). Skin types that burn easily and tan poorly are at greatest risk of developing AK, which have the potential for spontaneous regression with reduction in UV exposure and reappearance on resumption of UV exposure. Eventually lesions will become permanent (202).

Evidence that AK are the premalignant precursors of SCC, but not BCC, includes shared risk factors, a histologic continuum, and the presence of similar molecular genetic alterations in both AK and SCC (181). Approximately 40–50% of Australians age 40 yr or older have at least one AK, with an average of six to eight, and this incidence increases with advancing age (181,203,204). Other studies from Australia and the United States have reported AKs in 11–26% of the population (181). The rate of SCC in the population is much lower than the rate of premalignant AK, suggesting that not all AK progress to SCC (205). Presence of AK serves as a major risk factor for increased NMSC risk. In a 5-yr longitudinal study, 60% of SCCs arose from a preexisting AK, while in a 10-yr follow-up the rate of malignant progression to SCC increased 6–10%, or 1 per 1000 per year in individual lesions (203). A study from Arizona in individuals with >10 AK reported a cumulative probability of 14% for developing an SCC within 5 yr (204). While most AK do not progress to SCC, it is believed that AK represents SCC *in situ* at its earliest stages (206).

### *Treatment for IEN of Skin: Clinical Trials*

AK is the only precancerous skin lesion that can be treated with Food and Drug Administration (FDA)–approved drugs. Approved therapies include the antimetabolite 5-fluorouracil, photodynamic therapy with the photosensitizer aminolevulinic acid, diclofenac, and the creosote bush–derivative nordihydroguaiaretic acid (masoprocol).

Each of these agents is applied topically and indicated only for the treatment of AK, not cancer-risk reduction, although reduction of AK is a sensible strategy for reducing the risk of SCC (181).

Clinical trials designed to demonstrate the effectiveness of these agents in treating IEN of skin are necessary to determine whether this is an effective strategy for reducing the burden of NMSC. Several high-risk populations suitable for such trials include patients with multiple AK and organ-transplant recipients. For example, topical diclofenac in hyaluronan gel was approved for treatment of AK based on reduction of AK lesions in defined areas (207,208). Frank SCC was the end point in a trial of oral vitamin A (204) in which 2297 participants with a history of >10 AK (and <2 prior SCCs or BCCs) were randomized to placebo or retinol for at least 3 yr. This study showed a hazard ratio of 0.74 (95% confidence interval, 0.56–0.99;  $p = 0.04$ ) for the development of a new SCC in retinol-treated patients compared with patients receiving placebo. Furthermore, in a subset analysis the risk of SCC was reduced 50% in participants randomized to oral vitamin A who had  $\geq 8$  freckles or moles. Trials such as these will be necessary to demonstrate both the effectiveness of new agents and the utility of treating skin IEN to decrease the burden of NMSC. If toxicity of a given agent is low, a net clinical benefit may be realized owing to reduction in morbidity and potential reduced mortality.

Many experimental agents are in clinical trials to study chemoprevention of skin cancer. Numerous trials are targeting the AK lesion directly, while some are directed toward preventing or treating skin cancers. These trials include topical, parenteral, and oral agents. In a double-blind, placebo-controlled phase 2 study, topical colchicine (1% gel) resulted in complete regression of recurrent AK in 7 of 10 patients (209). In a phase 2a study (210), 48 patients with at least 10 AK on their forearms received topical difluoromethyl ornithine ([DFMO], an inhibitor of ornithine decarboxylase) on either the left or right forearm and placebo ointment on the other forearm twice daily for 6 months. DFMO reduced the number of AK 23.5% on treated forearms. Levels of the polyamine spermidine in biopsy samples were used as a biomarker of DFMO activity, and spermidine suppression was directly related to AK reduction; however, inflammatory reactions were seen in some of the DFMO-treated arms. A follow-up phase 2b study (150 patients) is ongoing. This study, in patients with moderate to heavy AK (>3 AK per forearm), is designed to assess additional biomarker end points and potentially confirm the results of the published trial. This study will also determine whether the irritation reported by subjects in the previous DFMO study can be reduced or eliminated by concurrent use of a topical corticosteroid.

Several other NCI-sponsored NMSC prevention trials are ongoing. A phase 2 randomized, double-blind, placebo-controlled trial is studying topical green tea extract (polyphenon E) in patients with at least two clinically and histologically confirmed actinic keratoses on each arm. This study is designed to determine the ability of polyphenon E to cause complete regression of AK, as well as effects on biomarkers and treatment duration. In another trial, the effect of oral fenretinide on clinical AK is being studied with a large, phase 2 randomized, double-blind, parallel-group design. This dose-finding trial is powered to determine whether fenretinide can decrease the number of clinical AK by at least 80% in patients with the presence of at least 15 AK. This study will also evaluate biomarkers and the pharmacokinetics of oral fenretinide. A phase 2 to 3, randomized, placebo-controlled, double-blind, multicenter study of

the effect of oral celecoxib on AK prevention/regression is expected to accrue 300 patients.

While most current chemoprevention studies are targeting AK, at least two ongoing phase 3 studies are designed to determine the effect of chemopreventive agents on frank NMSC. Following on positive phase 2 results (211), the North Central Cancer Treatment Group is running a phase 2/3 randomized, placebo-controlled study of acitretin in patients receiving solid-organ transplant who had multiple prior NMSCs. This study is expected to accrue 110 patients and will evaluate human papilloma virus (HPV) as a possible etiologic factor. Treatment is expected to last 2 yr, with patients evaluated every 6 mo. A randomized study of oral DFMO in patients with previously treated stage 0, I, or II BCC or SCC is expected to accrue 334 patients. Patients are evaluated every 6 mo, and treatment is expected to extend for 3–5 yr.

### *Future Therapies: Molecularly Targeted Agents*

Advances in human genome research and the advent of new technologies in gene analysis add an exciting dimension to this field. As selective agents are developed for specific molecular targets, individual tailoring of prevention strategies based on genetic analysis may become possible (212). Preclinical reports on new agents appear regularly in the literature. Molecular targets include cell-cycle and apoptosis regulators such as p53, second-messenger systems linked to cell proliferation such as the mitogen-activated protein kinases (MAPK) and AP-1 complexes, and inflammatory mediators such as COX. Agents that selectively target these pathways may be useful chemopreventive agents. Figure 4 shows the AP-1 signal transduction pathway and potential targets for cancer chemoprevention.

Apigenin, derived from chamomile, inhibits ornithine decarboxylase and stimulates the p53-p21/waf1 response pathway in mouse keratinocytes. This compound has been shown to inhibit UV-induced skin tumorigenesis when applied topically to mice (213,214). Chemopreventive nonsteroidal anti-inflammatory drugs (NSAID) exert their activity mainly through inhibition of COX. The COX-2 selective inhibitor celecoxib, as well as the nonselective COX inhibitor indomethacin have been shown to reduce UV-induced skin carcinogenesis in a mouse skin model (215). Green tea polyphenols, including epigallocatechin gallate, are some of the most-studied chemopreventive compounds (216). Topically applied extract has been shown to inhibit UV-induced skin carcinogenesis in a mouse skin model (217). In this study, the extract was applied topically to the backs of mice after UV irradiation. The mechanism of photocarcinogenesis inhibition was distinct from a simple sunscreen effect or inhibition of photoimmunosuppression. In support of this, others (218) have shown that the compound blocks AP-1 activation by inhibiting phosphorylation of p38 MAPK, an important step in UV-induced skin carcinogenesis. Other agents that appear to block these pathways include perillyl alcohol (219), silymarin (220), and salicylates such as aspirin (221).

### **Colon Cancer**

Colorectal adenocarcinoma is the second leading cause of cancer death in the United States, with year 2001 estimates of 135,400 new cases and 56,700 deaths (222). Approximately 15% of individuals afflicted with the disease belong to clinically identifiable high-risk groups including familial adenomatous polyposis (FAP) and hereditary nonpolyposis syndromes (223). Most cases of colon carcinoma are sporadic (223).

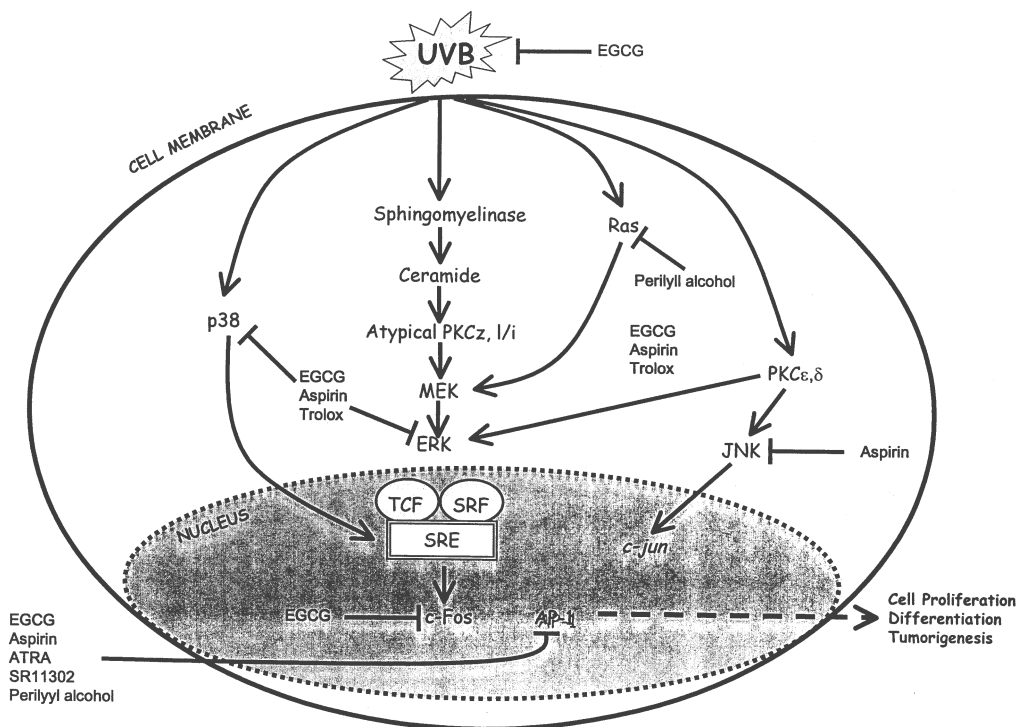


Fig. 4. AP-1 signal transduction pathway and points of intervention. UVB radiation elicits a complex cascade of molecular and cellular events. A series of enzymatic events results in activation of the transcription factor AP-1, which is responsible for transactivating numerous genes known to be involved in the process of carcinogenesis. Agents including EGCG, perillyl alcohol, trolox, and aspirin show promise as chemopreventative agents by blocking transactivation activity of AP-1 at various points within the signaling cascade.

During the last two decades, multidisciplinary research in epidemiology and molecular biology in vitro and in vivo has contributed greatly to understanding the etiology of colon carcinoma; more important, it has enabled design of preventive strategies. Risk factors for colon cancers have been identified and, significant progress has been made in understanding the process of colorectal carcinogenesis. Transition of a benign adenomatous polyp to a malignant lesion can occur over decades. It is estimated that 10–20 genetic events can occur in the time period between initiation and development of cancer, affording numerous opportunities for early diagnosis and intervention.

### Primary Prevention

Diet has been shown to play a role in risk of colorectal cancer, allowing preventive measures that are noninvasive and easily implemented. For example, reducing dietary intake of red meats and grilled meats can decrease risk three- to fourfold (224–227). The effect of a high-fiber diet has been a subject of intense study over the past two decades. Unfortunately, the results from both case-controlled and observational studies have been controversial. Studies examining the effect of cereal fiber (20–25 g/d for 2 to 4 yr) did not show an effect on adenomatous polyp recurrence (228–230).

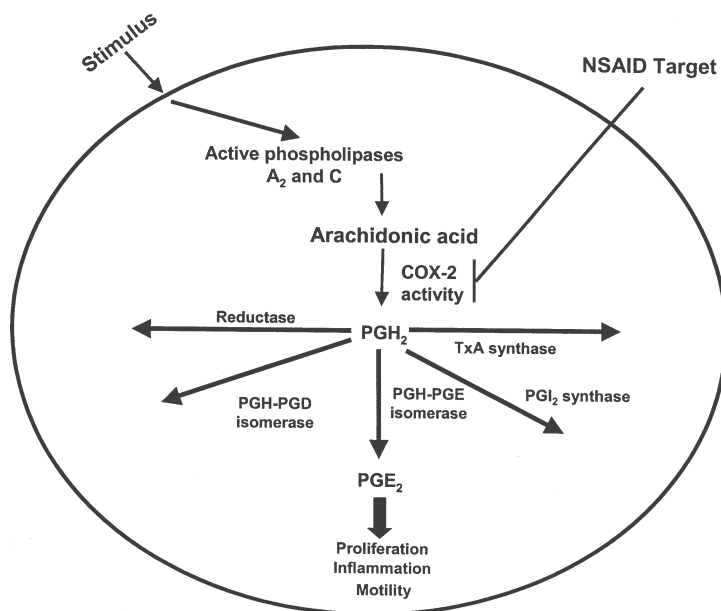


Fig. 5. Biosynthesis of prostaglandins. Membrane phospholipid is converted to arachidonic acid via  $\text{PLA}_2$ . Subsequently, arachidonic acid is converted to  $\text{PGH}_2$  through a two-step process that involves COX activity to convert arachidonic acid to  $\text{PGG}_2$  followed by a peroxidase reaction that is also catalyzed by COX to produce  $\text{PGH}_2$ . Further metabolism is catalyzed by tissue-specific isomerases. Effects downstream of COX-2 include cell proliferation and inflammation.

The National Polyp Prevention Trial, which enrolled 2079 subjects with a history of resected polyps, also failed to show a positive effect of a high-fiber diet (231). In addition, studies at the Arizona Cancer Center randomized 1429 subjects with a history of polyp resection to receive either a low- or high-fiber diet for a 3-yr follow-up period (232). At the 3-yr endoscopy, no significant difference in recurrence of polyps was observed between the treatment groups. Other factors that may influence risk of colon cancer include smoking and physical activity (233–235).

### COX-2 and Carcinogenesis

The COX-1 and -2 enzymes catalyze the conversion of arachidonic acid to prostaglandins (Fig. 5) (236,237). Prostaglandins and other products of arachidonic acid metabolism, including thromboxane and 15-hydroxy-eicosatetraenoic acids, belong to the eicosanoid family of fatty acid molecules, which are known to regulate numerous physiologic processes, such as the inflammatory response and other immune response modulators (238,239), ovulation (240,241), and mitogenesis (242). In the pathway of prostaglandin synthesis, several points are opportunities for intervention. In the first step, phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) catalyzes conversion of membrane phospholipid to arachidonic acid, which is converted to prostaglandin  $\text{H}_2$  ( $\text{PGH}_2$ ) through a two-step process that involves COX activity to convert arachidonic acid to  $\text{PGG}_2$  followed by a peroxidase reaction that is also catalyzed by COX to produce  $\text{PGH}_2$  (243,244).

There are two known isoforms of COX enzymes. The COX-1 isoform is a membrane-bound hemoglycoprotein that is constitutively expressed in the endoplasmic reticulum in most cells and is responsible for local prostaglandin synthesis. The COX-2 isoform is primarily inducible, although low basal expression is apparent in some types of tissues (245).

COX-2 inhibitors have shown great promise in the treatment of IEN. The specific mechanisms mediating the antineoplastic effects have not been elucidated, however. Several mechanisms of action have been proposed including inhibition of cell growth (246–248), induction of apoptosis (249–251), and inhibition of prostaglandin synthesis (252).

### *COX-2 In Vitro and Preclinical Data*

Overexpression of COX-2 has been documented in human colorectal adenomas and cancers, but not in normal-appearing mucosa (253). The chemopreventive effects of COX inhibitors on the development of colorectal cancer are being studied. Transgenic animal models have been extremely valuable in investigating the role of COX-2 in the pathogenesis of colorectal cancer. Using *Apc* delta716 knockout mice, which carry a mutation of the *APC* gene that results in spontaneous adenoma formation in the small intestine, a cause–effect relationship was established between COX-2 overexpression and GI tumor incidence (254). These studies suggested that suppression of a single allele of the *cox-2* gene reduced the number of intestinal polyps by 66%, and suppression of both alleles resulted in a reduction of 86% (255,256). Furthermore, treatment of COX-2-expressing azoxymethane-treated rats with oral celecoxib suppressed formation of colorectal tumors by >90% compared with a suppression of 40–65% after administration of a nonselective COX inhibitor (256,257).

Data collected prospectively have shown that long-term chronic administration of aspirin and other COX-1/-2 inhibitors has been associated with a reduction in the incidence of colorectal adenomas, cancer, and cancer mortality by 40–50% (258–261). Furthermore, case-control studies have concurred with these data (262–264). Clinical studies showed that oral sulindac, a commonly used NSAID, was associated with a reduction in the number and size of adenomas in patients with FAP (265,266). Consequently, the FDA granted approval of celecoxib, the selective COX-2, for treatment of FAP. The pivotal trials of celecoxib for the treatment of FAP enrolled 77 patients who were randomized to receive either placebo or celecoxib (100 mg or 400 mg twice daily) for 6 mo (267). The primary efficacy end point was the percentage change in the number of colorectal adenomas >2 mm at 6 mo. A 4.5% reduction was seen in the placebo group, an 11.9% reduction in the celecoxib 100-mg group, and a 28% reduction in the celecoxib 400-mg twice daily group. The difference between the celecoxib 400-mg twice daily group and the placebo group was statistically significant ( $p = 0.003$ ). The incidence of adverse events was similar among the groups and consisted primarily of diarrhea, dyspepsia, fatigue, upper respiratory infections, and rash.

The results from the pivotal trial of celecoxib in FAP support the further investigation of COX-2 inhibitors in an overall strategy for chemoprevention colorectal tumors in other populations at risk, including persons with sporadic adenomatous polyps. As shown in Table 1, celecoxib is being tested in several clinical trials for the prevention or recurrence of colorectal adenomas. Two studies are being conducted under the sponsorship of the Division of Cancer Prevention, NCI. One is investigating two dose levels of celecoxib vs placebo with the end points of 1- and 3-yr colonoscopy examinations. A second phase 3 clinical trial used a factorial design to study celecoxib vs selenium vs

the combination of celecoxib and selenium vs double placebo. Recurrence rates of adenoma will be evaluated at the 3-yr colonoscopy end point. The results of these trials will not be available for several years, but they could establish COX-2 inhibitors as important components of the management of colorectal adenomas and the prevention of colon cancer.

### *Other Agents*

Over the past decade, several clinical studies have been conducted testing various natural products as potential colon cancer preventive agents, including  $\beta$ -carotene (268,269), vitamin E (270), vitamin D (271,272), calcium (273–275), and selenium (276). Of these, two randomized studies demonstrated a statistically significant effect on polyp recurrence. One study showed a 17% decrease in recurrence of polyps in participants receiving calcium carbonate supplement compared with placebo (277,278). In addition, a 29% reduction in polyp recurrence was observed in another study testing the effects of selenium supplementation on cancer risk (279).

Calcium may interfere with colon carcinogenesis by binding bile acids and fatty acids in the bowel lumen or by directly inhibiting proliferation of cells in the colonic epithelium (274,280–282). In vivo animal studies have shown a decrease in hyperproliferation in the colon and reduction of tumor formation with calcium supplementation (282). In the clinical study, the protective effect was observed in the first year of observation in some patients, suggesting that calcium may act at a relatively early stage in the process of colon carcinogenesis (274,281).

### *Lung Cancer*

Epidemiologic studies conducted throughout the past 50 yr have helped identify the deleterious effects of smoking and other forms of tobacco use. These studies have clearly established that tobacco use is responsible for most primary malignancies of the lung (283–289). Tobacco use is also a great risk factor for development of cancers of the oropharynx, larynx, and esophagus (290,291) in the United States, and approx 30% of all cancers of the pancreas (292), kidney (293,294), urinary bladder (295,296), and uterine cervix (297, 298). The incidence of lung cancer continues to have an impact of epidemic proportion on the health of people worldwide. In 2001, approx 169,500 new lung cancer cases were diagnosed in the United States alone, and deaths from lung cancer account for >25% of all cancer mortalities. While more American women and men will be diagnosed in the year 2001 with breast or prostate cancer, respectively, lung cancer will remain the leading cause of cancer mortality for both sexes (222). Although lung cancer incidence is significantly higher in men than in women, since the 1950s, a striking 600% increase in lung cancer incidence and mortality has been seen in women due to the increase in smoking prevalence by women who had begun smoking earlier in the last century (299,300).

Current approaches for lung cancer therapies have not successfully impacted long-term survival. As is true with most cancers, the key to improvement of morbidity and mortality will rely on a combination of early detection, development of novel, targeted therapies for early and late-stage disease; and identification of chemopreventive agents in high-risk populations, such as former smokers.

### *Etiology and Prevention*

Tobacco-exposed populations continue to provide opportunities to characterize somatic mutations that occur in the early stages of carcinogenesis and to identify genetic

inherited susceptibility and resistance traits to lung and other types of cancers related to tobacco use. Fortunately, despite their complexity, recurrent chromosomal aberrations are beginning to emerge. For example, aberrant methylation of the promoters of two genes, *p16* and *O*<sup>6</sup>-methyl-guanine-DNA-methyltransferase, has been detected in DNA isolated from 100% of sputum samples from patients with squamous cell lung cancer up to 3 yr before diagnosis (301). The protein hnRNP B1 is involved in mRNA processing; its expression has been reported through bronchial biopsy in 64% of bronchial dysplasia samples compared with 0% in normal bronchial epithelium (302,303).

Occupational exposures have been estimated to account for up to 20% of all lung cancers (304,305). Chemical and physical agents that have been identified as lung carcinogens in the workplace include arsenic, asbestos, ethers, chromium, cadmium, nickel and polycyclic aromatic hydrocarbons, radon, and vinyl chloride. More recent studies have identified additional occupational risks, including materials used in the manufacturing of computers and rubber/plastics (306).

### *Treatment of Early Lung Carcinogenesis*

Efforts focusing on lung cancer chemoprevention have been based on two premises. The first is that the process of lung carcinogenesis requires multiple steps. The second is the paradigm of field carcinogenesis that suggests that the aerodigestive epithelium, which includes the respiratory epithelium, has been diffusely exposed to the carcinogens. Epidemiologic studies conducted in the 1950s support the premise of field carcinogenesis by demonstrating that multiple, distinct invasive cancers were often apparent within lung cancer surgical specimens (307). The multistep nature of lung carcinogenesis suggests that the premalignant stages offer opportunities to use agents to prevent or reverse the disease process. It is of interest that histologically normal-appearing bronchoepithelium of smokers can harbor alterations that have been associated with lung carcinogenesis (308).

Because 40–50 million former smokers live in the United States, strategies to prevent primary lung cancers are the subject of intense clinical study. Furthermore, in addition to using development of primary or secondary cancer as end points in chemoprevention studies, potential biomarkers are known. Intermediate markers of lung carcinogenesis include histologic lesions such as bronchial metaplasia and dysplasia (309–311) and genetic abnormalities such as loss of heterozygosity at chromosomes 3p and 9p (312–314). Other biomarkers of lung carcinogenesis being evaluated include mutations in *p53*, the TSG (315); the oncogene *ras* (316–318); epigenetic abnormalities such as hypermethylation of the promoter region of receptor molecules, including EGFR (319,320); the retinoid receptors RAR and RXR (321,322); and enzymes that regulate synthesis of prostaglandins (323).

### THE RETINOIDS

Previous chemoprevention studies evaluating retinoids as lung cancer chemopreventive agents provided insight into the importance of rigorous testing of hypotheses derived from epidemiologic and laboratory data in the setting of large randomized clinical studies, taking into account confounding factors that affect the population of study. Both retinol (vitamin A) and its precursor,  $\beta$ -carotene, have had significant epidemiologic support as potential chemopreventive agents (324,325). Furthermore, in vitro and in vivo data support cancer inhibitory activity for retinol and retinol analogs (322, 326,327).



In a clinical study testing the efficacy of retinyl palmitate vs observation after standard, front-line therapy for stage 1, non-small cell lung cancer (NSCLC), a median follow-up of 46 mo showed a higher incidence of development of a second primary tumor in the placebo group (48%) compared with the treatment group (39%) (328). Other, uncontrolled studies showed promise for retinoids as effective chemopreventive agents for lung cancer (329). The Carotene and Retinol Efficacy Trial (CARET) evaluated  $\beta$ -carotene and retinol in two populations at risk for lung cancer, including asbestos workers and cigarette smokers. CARET was a randomized trial with a  $2 \times 2$  factorial design. Statistical analyses showed that the  $\beta$ -carotene-supplemented cohort of current smokers had a 28% increase in the rate of lung cancer (330–332). This finding was confirmed by a study conducted in Finnish men using  $\beta$ -carotene and vitamin E in the Alpha-Tocopherol Beta-Carotene study, which showed an 18% increase in lung cancer and an 8% increase in total mortality (333,334). It has been hypothesized that oxidation products of  $\beta$ -carotene in the presence of carcinogens associated with lung cancer may have synergistic effects that increase cancer risk. In vitro and in vivo studies have supported this hypothesis (335).

#### SELENIUM

In the United States, lower mortality for a number of cancers, including lung cancer, has been associated with geographic regions with moderate to high selenium in forage crops (336). In addition, subset analyses of the Nutritional Prevention of Cancer Trial showed that selenium reduced the incidence of lung cancer in persons with a relatively low baseline plasma selenium concentration (131,132). These data led to initiation of an additional randomized, placebo-controlled clinical study examining the chemopreventive effect of daily selenium supplementation in former smokers.

#### INHIBITORS OF PROSTAGLANDIN SYNTHESIS

COX-2 has shown promise as a target for chemoprevention of lung carcinogenesis and as treatment of more advanced lung cancers. COX-2 has been shown to be an inducible enzyme expressed in a significant percentage of lung adenocarcinomas but is not highly expressed in normal bronchial epithelium (337–339). Selective COX-2 inhibitors including celecoxib are under clinical investigation for their use as chemopreventive agents for lung cancer. Other agents that act by inhibition of the prostaglandin synthesis pathway that have demonstrated antiproliferative effects in the in vitro models of lung carcinogenesis are the inhibitors of lipoxygenase (340,341).

#### EGFR INHIBITORS

EGFR is a dimerized cell membrane receptor involved in regulation of cellular growth signal transduction pathways that mediate cell proliferation, differentiation, and apoptosis. While present in normal epithelial cells including skin, corneal, and GI epithelium, EGFR is overexpressed in a number of cancers including NSCLC. EGFR is overexpressed in preneoplastic lesions and carcinomas *in situ* in the lung (342).

Development of orally bioavailable formulations of EGFR inhibitors with favorable toxicity profiles has afforded the opportunity to target EGFR in a population that has undergone surgical resection of lung cancers to reduce the incidence of development of second, primary aerodigestive tumors. Measurement of expression of EGFR and factors involved in the process of carcinogenesis in the lung responsible for regulation of

cell growth, differentiation, and apoptosis can be used as surrogate end point biomarkers to help evaluate efficacy of chemopreventive agents.

### **Screening and Early Detection of Lung Cancer**

The highly lethal nature of lung cancer can, in large part, be related to the challenge of early diagnosis. Lung cancers are often not symptomatic and are therefore not detected until an advanced stage of disease at which point cure is unlikely. Furthermore, owing to a biologic propensity for early micrometastases, aggressive nature of the tumor type, and inherent resistance to drug therapies, prognosis of early stage lung cancers is relatively poor compared with other solid tumors. Prognosis has been shown to be significantly better in patients diagnosed with stage I disease with 5-yr survival of 61–67% compared with 1–13% 5-yr survival for patients diagnosed with regionally advanced (stage III) and metastatic (stage IV) disease (343,344).

The ideal means for reducing lung cancer incidence is through prevention efforts by eliminating exposure to carcinogens and by developing effective screening strategies that would allow for the detection of stage I disease. The earliest method implemented was traditional chest radiography with or without cytologic analyses of sputum samples. More recent screening efforts have used more-sensitive imaging technologies, including helical low-dose computed tomography (CT) scanning. Identification of molecular markers in sputum associated with carcinogenesis in the lung has added promise to screening strategies. Despite efforts that had begun in the 1950s, including 10 prospective clinical studies using chest radiographs and/or sputum cytology, no standard recommendations exist for lung cancer screening. Both the Memorial Sloan Kettering Lung Project (345,346) and the Johns Hopkins Lung Project (347) were NCI-sponsored efforts that evaluated the efficacy of screening for lung cancer in male smokers by comparing annual chest radiographs with sputum cytology in combination with radiographs. Both studies showed no difference in patient outcome with the addition of sputum cytology to traditional imaging. Another study conducted in Czechoslovakia randomized >6000 male smokers to the study group, which underwent chest radiographs and sputum cytology at 6-mo intervals for 3 yr, or the control group, which underwent annual radiographs and sputum cytology for 3 yr. After the 3-yr study period, each group underwent annual chest radiographs for an additional 3-yr period. Survival was improved in the study group; however, the differences were not statistically significant (348–350). Although the original findings were discouraging, subsequent interpretations of the studies revealed that confounding factors not taken into consideration caused an imbalance of the study and control groups. The confounders included genetic predisposing factors, environmental exposures (including asbestos and radon), and influence of overdiagnosis bias (i.e., where clinically nonrelevant lung cancers are identified in a population with competing morbid conditions), which may provide an explanation for the trend toward increased mortality in the screened groups. Although subsequent analyses did not show significant reductions in mortality, improvements were seen in the outcomes of lung cancer stage distribution and respectability (351,352).

More sensitive screening techniques are being evaluated in clinical studies. The Early Lung Cancer Action Project (ELCAP) was designed to define curability rate based on the size of nodule at the time of detection. This study used helical, low-dose CT imaging of the chest in a nonrandomized cohort of 1000 smokers aged 60 yr or older (353). Data from this study have reported that helical, low-dose CT scanning is more effective

than chest radiographs in detection of noncalcified lung nodules. ELCAP included the use of an additional high-resolution CT scan for assignment of a follow-up program of reimaging of nodules vs proceeding directly to biopsy. Implementation of screening for lung cancer using these more sensitive detection techniques will require validation of larger and broader study populations.

### ***Molecular Markers of Lung Carcinogenesis***

Integration of techniques measuring molecular markers of early lung carcinogenesis will be critical for improving prognosis for patients with the disease. Specific genetic and epigenetic alterations in the bronchoepithelium that have been shown to have predictive value for detection of early lung cancer include proteins that are differentially expressed in normal vs preneoplastic bronchoepithelium; mutation in genes involved in regulation of cell proliferation, differentiation, and apoptosis; and alterations in genetic and cellular elements that influence the transformation from normal to malignant tissue. Candidate biomarkers that show promise include methylation status in the promoter regions of the TSG gene *p16* (354,355) and *O*<sup>6</sup>-methyl-guanine-DNA-methyltransferase (356), which is an enzyme involved in metabolism of carcinogens. Studies have shown that both genes were hypermethylated in 100% of squamous cell lung cancers up to 3 yr before diagnosis (301). The mRNA processing protein hnRNP has been shown to be inappropriately expressed in biopsy tissue with bronchial dysplasia compared with normal bronchial epithelium (302).

### ***Barrett's Esophagus and Esophageal Cancer***

In the last decade, the incidence of esophageal adenocarcinoma has increased at a disproportionately rapid rate (357). The most well-characterized risk factors include gastroesophageal reflux disorder (GERD) and its sequella, Barrett's esophagus, which is considered to be a precursor condition manifested by the replacement of normal esophageal epithelium with a columnar type of metaplastic intestinal epithelium predisposed to malignancy (358,359). Barrett's esophagus is frequently associated with high-grade dysplasia and aneuploidy in esophageal epithelium (360–363). The condition develops from chronic, severe gastroesophageal reflux. Patients afflicted with Barrett's esophagus have a 30- to 40-fold increased risk of developing of esophageal adenocarcinoma (364–366). Barrett's esophagus can be arbitrarily divided into several categories. Long-segment and short-segment types are defined by the length of metaplastic tissue being >3 cm or <3 cm, respectively. Barrett's esophagus is considered microscopic when very small isolated areas of specialized intestinal metaplasia exist at the esophagogastric junction. The increase in risk of developing adenocarcinoma is significantly less with short-segment Barrett's esophagus (10%) compared with long-segment Barrett's esophagus (30–40%). Regular screening in patients with short-segment Barrett's esophagus is controversial.

Postsurgical morbidity is high for esophageal cancer, and survival is not favorable (13–30% for 5 yr) (367–369). No effective treatment modalities are available, and chemopreventive strategies and treatments for the precursor condition are needed.

### ***Strategies for Preventing Early Stage Carcinogenesis***

#### ***Etiology and Prevention***

Patients with Barrett's esophagus acquire columnar metaplastic tissue in the lower region of the esophagus as a consequence of long-term GERD. Therefore, conditions

that cause an increase in the reflux of gastric acid into the esophagus represent a risk factor. These conditions can include delay in esophageal acid clearance time, decreased resting pressure of the cardiac sphincter, hiatal hernia, and esophageal injury. Other more rare etiologic features include bile reflux after gastrectomy and congenital predisposition; however, specific gene mutations associated with an increased risk of Barrett's esophagus have not been identified.

DNA ploidy is often used as a marker of tumor progression in Barrett's esophagus-associated neoplasia. Aneuploidy was seen in 9 of 13 tissues isolated from patients with Barrett's esophagus (370–372), and expression of cancer-related protooncogenes and TSGs has been reported in patients with Barrett's esophagus-associated neoplasia. Thirty-one percent of tissues isolated from human esophageal tumors overexpress EGFR (373). Alterations in expression of oncogenes including *src* and *bcl-2* (374–376) and the adhesion molecule E-cadherin have been reported (377,378).

### *COX Inhibitors*

In vitro and preclinical data have suggested that COX inhibitors may be effective in treating of Barrett's esophagus and preventing esophageal carcinomas. Nonspecific COX-1 and COX-2 inhibitor, aspirin, caused dose- and time-dependent growth inhibition of an esophageal adenocarcinoma cell line (379). The COX-2-specific inhibitor, NS398, has been shown to upregulate expression of genes that regulate apoptosis. In vitro, the COX-2-specific inhibitor induced apoptosis in several esophageal adenocarcinoma cell lines through a cytochrome-c-dependent pathway. Furthermore, a direct relationship was observed between apoptosis and the level of COX-2 expression. Caspase-9 and caspase-3 were activated, and addition of a caspase inhibitor reversed the apoptotic effect of the COX-2 inhibitor (380). These data suggest that COX-2-specific inhibitors may be promising for chemoprevention and treatment of esophageal adenocarcinoma.

Studies examining tissue collected from esophageal pinch biopsy specimens showed increased expression of COX-2 protein in 41% of the Barrett's esophagus tissue biopsy specimens with and without dysplasia. COX-2 expression was not detected in adjacent normal tissue. These data suggest that COX-2 may have a role in the early stages of development of adenocarcinoma of the esophagus (381). A clinical study is evaluating the efficacy and safety of celecoxib in patients with Barrett's esophagus.

### ***Tumor Immunology and Carcinogenesis***

The host's ability to fight infections is critical to prevent succumbing to infection. The same is true for cancer cells; however, because tumor cells are derived from the host, these cells do not present to the immune system as foreign bodies, thus making cancer cells poor immunogens. It is crucial that the immune system detect and mount an effective antitumor response against cancerous cells to prevent the development of tumor formation. Elucidation of tumor immunology has been of paramount importance in cancer research. New therapeutic techniques are examining the possibility of harnessing and enhancing the immune system to prevent cancer and treat carcinogenesis.

The ability of a host to rid a foreign pathogen from the body depends on two major components: the innate and adaptive (acquired) immune systems. Physical barriers such as the skin, mucosa, and tears provide the first line of defense against the outside environment (382). If these barriers are broken, biochemical barriers such as lysozyme and serum complement provide protection from pathogen invasion and infection. Inflam-

matory cells, such as macrophages and neutrophils, are important cellular components of the innate immune response and serve as a bridge between the innate and adaptive immune responses (383).

The adaptive immune system serves to mount specific antigenic responses, discriminates between foreign antigens (Ags) and self-Ags, and possesses immunologic memory to respond faster and more effectively on a second Ag challenge. The adaptive immune response is composed of two parts: the humoral part, consisting of B-lymphocytes that produce and secrete Abs; and the cellular part, in which the activation of tumor-specific T lymphocytes is necessary for tumor-cell lysis. To complement the antigenic diversity that exists in nature, the immune system is composed of >1 million specificities for each B-cell and T-cell repertoire due to multiple gene segment rearrangements of B-cell receptors (immunoglobulins) and T-cell receptors (TCRs) (384). The successful induction of the adaptive immune response will result in the production of antibodies and T lymphocytes that are specific for the invading pathogen.

### ***Humoral Immunity***

Antitumor humoral responses are composed of Abs specific for tumor Ags. Tumor-specific Abs bind to Ags on the surface of tumor cells such as gp100 (melanoma Ag) and an EGFR, Her-2/neu (adenocarcinomas). The circulating Abs bind to Ags on the surfaces of tumor cells, flagging cells for destruction by immune cells such as macrophages and natural killer (NK) cells. Cell death may result from either Ab-dependent cellular cytotoxicity (ADCC) or complement-mediated lysis. ADCC is a result of Abs coating target cells. Cells bound by Abs are detected and killed by immune cells, such as NK cells possessing Ab receptors (Fc receptors) (385,386). In effect, Abs direct Ags specificity, and effector cells, which are not Ag specific, perform cytotoxic functions. Complement-mediated lysis of Ab-coated cells results from a concerted effort of several complement proteins (components of the innate immune system) that bind to both the Abs and the surface of cells, ultimately resulting in the formation of complexes capable of puncturing cellular membranes.

### ***Cellular Immunity***

The generation of specific cellular immunity requires Ag presentation to T lymphocytes by antigen-presenting cells (APCs). Dendritic cells (DCs), macrophages, and B-cells serve as APCs to activate T-cell responses. APCs ingest foreign bodies, digest Ag, and present antigenic peptides to T cells in association with class I and class II major histocompatibility complex (MHC) proteins on the surface of APCs (387,388). TCRs on the surface of CD4<sup>+</sup> T-helper lymphocytes recognize peptides presented by MHC class II, while CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) recognize peptides presented by MHC class I. Peptides derived from proteins outside the cell that have been engulfed by APCs are presented in association with MHC class II molecules, whereas peptides presented by class I molecules are derived from within the cell. Tumor-derived peptides can be presented through the MHC class I pathway by tumor cells. On MHC–TCR interaction, the TCR is responsible for the specificity of the response. The binding of a variety of costimulatory and adhesion molecules between both APC and the T cell further activate the T cell, resulting in T-cell effector functions, such as cytokine secretion and lysis of target cells.

CD4<sup>+</sup> T cells are activated by APC presenting MHC class II–peptide complexes. CD4<sup>+</sup> T cells can be divided into two subsets, Th1 and Th2 cells, based on the cytokines

that cells secrete (reviewed in ref. 389). Th1 cells secrete interleukin-2 (IL-2) and interferon- $\gamma$  and help to drive the immune system toward a cellular response. Th2 cells produce IL-4, IL-5, IL-6, and IL-10, stimulating a humoral response.

CD8<sup>+</sup> T cells (CTLs) become activated after MHC class I–peptide Ag presentation and costimulation by APCs. Once activated, CTLs no longer require costimulation by APCs to kill target cells. Activated CTLs induce cell death through granzymes (lytic granule enzymes), perforin, and the Fas–Fas ligand apoptotic pathway (390). CD8<sup>+</sup> cells are a major component of antitumor responses, and several cellular immunotherapy protocols aim at activation of this subset of lymphocytes.

### ***Adaptive Immune Response and Cancer***

Most tumor cells express self-Ag, which makes tumor cell detection by the immune system difficult. T cells are educated in the thymus early in life and thymic education plays an important role in the generation of T cells that are tolerant to peptides derived from self-Ag (391). Thymocytes that interact with MHC–peptide complexes on the surfaces of thymic epithelial cells are positively selected for the ability to recognize self-MHC molecules. Thymocytes that bind with a strong avidity for MHC–self-peptide complexes displayed by thymic DCs or macrophages are negatively selected in the thymus and forced to undergo apoptosis (392).

Autoreactive T cells can be detected in the periphery, and can initiate an immune response despite a low affinity for MHC–self-peptide complexes (393–395). Tumor cells frequently overexpress self-Ag; however, T cells with a strong affinity for self-Ag are deleted during thymic education. Nonetheless, it is clearly evident that T cells with a low affinity for self-peptides are not capable of sufficiently resolving tumor burden. Antitumor T cells have been isolated from peripheral blood, tumors, and tumor-draining lymph nodes (396–398). Methods have been developed for the isolation of tumor-infiltrating T cells specific for tumor antigens (tumor-infiltrating lymphocytes [TILs]) from melanoma lesions, activation, and expansion of these T cells in vitro, followed by reinfusion of these autologous TILs into patients (399). Tumor regression was observed in patients that received both TILs and IL-2 administration. These studies further demonstrate the importance of using immunotherapeutic strategies aimed at the activation of T cells.

### ***Prophylactic Cancer Vaccines***

Several microbial and viral agents have been linked to the development of cancer. Infections with *Helicobacter pylori*, hepatitis B virus (HBV), and HPV have been linked to the development of gastric cancer, hepatocellular carcinoma, and cervical cancer, respectively. Attempts have been made to design and synthesize vaccinations that can protect high-risk populations from the onset of disease in an effort to stimulate the immune system and develop memory responses to protect against further Ag challenges. While many prophylactic approaches have been established in animal models (as reviewed in ref. 400), there is considerable promise for use in human studies and advances in this area are likely.

### ***Current Strategies for Induction of Tumor-Specific Immune Responses***

Once a tumor has been detected, various therapeutic approaches to eliminate disease are available to patients: surgical removal, chemotherapy, radiation, and immunotherapy. Numerous clinical trials use DCs to stimulate strong antitumor T-cell

responses. Additionally, several therapeutic tumor-specific Abs, approved by the FDA are efficacious in the treatment of various cancers.

Immunotherapeutic strategies that target the activation of specific antitumor T-cell responses by DCs are promising against cancer. The induction of a successful cell-mediated tumor-specific immune response relies on Ag capture, processing, and presentation by APCs to T-lymphocytes. DCs are considered the most effective, efficient, and potent APCs of the immune system (401,402). DCs have been used in numerous clinical trials for the treatment of cancer. Immunization of patients with DCs is considered nontoxic, because no dose-limiting toxicity has been observed (403). Large numbers of DCs can be generated *ex vivo*, cultured with tumor Ag, and readministered to patients. The induction of tumor-specific T-cell responses has been detected in patients who have received DC immunotherapy.

Initially, vaccination protocols involved DCs pulsed with synthetic HLA-binding peptides. Several other methods involving DCs for immunotherapeutic purposes have been investigated, including DC–tumor cell fusion, transfection of DCs with tumor RNA or viral vectors, and DC exposure to tumor apoptotic bodies or exosomes.

### ***Immunologic Markers and Prognostic Indication***

The most effective treatment for superficial bladder cancer and recurrent tumors is intravesical immunotherapy with bacillus Calmette–Guérin (BCG) (reviewed in refs. 404 and 405). Cytokines are proving to be beneficial prognostic indicators to determine the effectiveness of such BCG therapy. Urine samples collected either 6 h (for IL-8 assessment) or 12 h (for IL-18 assessment) after the first round of BCG administration were used to determine that high levels of urinary IL-8 and IL-18 correlated with an increase in disease-free survival (404). In another study, urine samples were collected from patients before and 6–8 h after six weekly doses of BCG. Patients with urinary IL-2 concentrations <27 pg/μmol of creatine were more likely to have tumor recurrence, compared with patients with high IL-2 concentrations (406).

The expression of IL-6 and the IL-6 receptor (IL-6R) was examined in oral SCC lesions from 86 patients (407). Overexpression of IL-6R was correlated with larger tumors, and the presence of IL-6 mRNA transcripts was associated with lymph node involvement, metastasis, and disease recurrence.

Increased serum IL-10 concentrations in patients with advanced NSCLC were correlated with a poorer prognosis and reduced survival (408). Patients with metastatic disease displayed significantly higher amounts of IL-10 compared with patients with undisseminated cancer. Serum IL-10 concentrations from patients who were unresponsive to treatment were significantly higher compared with those of patients who were responsive (complete or partial tumor shrinkage or stable disease). Taken together, these studies indicate that cytokine expression analyses can serve as useful immunologic markers for patients. These types of analyses may be useful for detection of disease progression and could potentially be used to customize treatment regimens.

### ***Early Detection of IEN***

### ***Proteomics and Cancer Therapeutics and Prevention***

The term *proteomics* was added to the emerging language of the genome era in 1994 to capture the growing research activities that were beginning to link the massive genome-sequencing effort to the technical challenges of studying how the protein products of the genome function together in a cell or a tissue and even in an

entire organism (409). Proteomics has since evolved as a term that is more generally recognized to mean the simultaneous or parallel study of the complement of proteins, referred to as the proteome, that are expressed at any given time by a cell using large-scale technologies for protein separation and identification. The term *proteomics* is often synonymous with functional genomics, whereas the large-scale study of protein structure has been associated with the term *structural genomics* (410,411).

Much of the focus in technology development for evaluating the proteome in clinical cancer applications lies in improving the ease of protein analysis in samples that contain a complex mixture of proteins, such as a blood sample, a cancer cell, or a tissue section (412,413). Efforts include miniaturization and resolution enhancement of gel-based technologies (414,415) incorporating signal-amplifying dyes (416) and radiolabel chemistries (417) that compare simultaneously two proteomes of interest (e.g., tumor vs normal cell, treated and untreated tumor cells). Most improvements in computational power over the past decade have contributed immeasurably to the ability to perform massive parallel analysis of proteins and genes derived from any format and, to date, remain one of the greatest informational challenges to the field.

### **Applied Proteomics**

As the technologies advance for the study of the proteome, much promise has been placed on the role of proteomics in solving critical issues in cancer biology and the diagnosis, treatment, and prevention of human tumors (412,413,418). In tumors, the cellular complement of proteins becomes improperly regulated either through mutations in genes or from improper interaction with other proteins and signaling molecules (protein:protein interactions) (419). Since proteins are the product of the genome and the work force of the cell, they have been used as markers in cancer diagnostics and prognosis (e.g., loss of expression [p53], altered expression [Her-2/neu]) and studied as candidate targets for therapeutic intervention (419,420). Proteins, particularly those that function at the cell surface (receptors) or those that are unique to cancer, are ideal targets for pharmaceutical intervention and, as such, knowledge of their expression and role in tumor biology has directly resulted in the growing number of successes for targeted therapies for human cancers (421–424).

It is widely envisioned that the clinical application of proteomic techniques will impact the care of the individual cancer patient. Early clinical studies are already demonstrating the value of profiling proteins in a complex mixture of tumor cells for monitoring drug responsiveness (425,426), for identifying new drug targets for prevention (427,428) and treatment (429,430), and for the detection and diagnosis of cancers (431–434).

### **Cancer Prevention and Treatment Response**

In spite of the overexpression of key growth factor receptors on particular premalignant and malignant cell types and the availability of targeted therapies, some lesions fail to respond to therapy, in part because the cell circuitry made up of complex protein:protein interactions is bypassed or disabled (419,420). It is believed that the neoplastic cell that is arrested or inhibited by a particular targeted therapy will have a particular sensitive proteome response after exposure to that drug. Conversely, those patients whose neoplasias have developed resistance will demonstrate an altered protein profile or altered proteome response to drug therapy that is indicative of drug resistance. Characterizing premalignant and malignant protein patterns after drug



delivery and monitoring in relation to treatment response (sensitive or resistant) holds promise for individualizing treatment choices based on *a priori* knowledge of the drug responsiveness of the cancer cell (435,436). Several ongoing clinical trials of novel agents that target tumor-associated proteins incorporate proteomic approaches to evaluate response to drug therapy by analyzing the change in protein patterns and the change in individual protein species that are coordinately regulated in the response and to develop algorithms based on particular proteome drug response patterns that predict drug sensitivity. Clinical studies that define the therapeutic response to individual agents based on proteome response will improve decisions regarding the drug selection for the individual cancer patient and the person at risk for neoplasia development. In parallel, proteome drug response analysis of this nature will facilitate more informed combining of drugs based on observed overlap in proteome responses including missed or evaded targets, and independent pathways such that combinations may give rise to improved drug performance characteristics in the premalignant and malignant cell type.

### **Drug Discovery**

In addition to identifying patterns or profiles that predict treatment response, proteomic approaches are actively being used to identify novel drug targets for development as new prevention and anticancer agents (437). Building on protein circuitry, active mapping programs are ongoing to link, in networks, individual proteins such that upstream and downstream regulators of tumor growth, angiogenesis, tumor movement, and invasion are being identified in attempts to characterize major protein regulators in a circuit or node and target these for drug development. In the pipeline of drug discovery, these key regulator proteins become candidates for intensive structural characterization and drug development.

### **Early Detection and Diagnosis**

In addition to an immediate impact in therapeutic monitoring and drug development, proteomic applications are showing similar clinical promise in the application areas of early detection and cancer diagnosis (418,427,428,431,432,434). For example, serum protein profiles generated using surface-enhanced laser desorption ionization-mass spectrography (SELDI-MS) technology were shown to reliably identify women who harbored all stages of ovarian tumors, including women with early stage I disease (433). Similarly, in two studies of men with prostate cancer, SELDI-MS-generated serum protein profiles were able to predict the presence of prostate cancer with a specificity and sensitivity comparable to or better than standard PSA testing (431,432). Further studies are required to confirm the suggestion that SELDI-MS technology may detect prostate tumors in a simple blood test 5 yr earlier than standard testing methods (432). Although to date few data have emerged evaluating proteomic approaches for neoplasia staging, differentiation, and prognostication, the clinical application of new proteomic technologies that are adapted to small biopsy specimens holds great promise for improved detection of early lesions and for determination of their clinical significance. With advances in technology and computational power to evaluate large panels of proteins that comprise a cell proteome, it is very likely that these tools will rapidly move beyond proof of principle and translate to approved medical applications as more clinical studies are completed that validate and expand the fields of use for these new technologies.

### ***Molecular Pathology: Karyometric Analysis for Early Detection of Progression to Malignancy***

Imaging techniques have been developed that can detect and objectively quantify subtle alterations in nuclear chromatin patterns that cannot be detected by standard histopathologic examination. The quantitative nature of the approach prevents observer bias from affecting the results. This technique has high precision and reproducibility in detecting subtle nuclear changes (87,88,438–440). Abnormalities in nuclear chromatin can be detected at an earlier stage of carcinogenesis than can be detected by histopathology. Therefore, further development and validation of this technique may have an impact on diagnosis of cancer and treatment at early stage disease with agents that target the initial events of carcinogenesis.

### ***Karyometric Features***

Nuclear images from normal reference tissue and abnormal tissue are segmented and digitized, and information on 93 karyometric features is extracted from the distribution of pixel gray values in the digitized images. These features incorporate a wide range of information. At the simplest level, measures of total optical density (OD), mean OD, variance of pixel OD, and total nuclear area constitute global measures summarizing the state of the entire nucleus. The relative frequencies of occurrence of pixels in bins of the OD histogram provide a second set of features that vary as a function of both the amount and distribution of chromatin. The third major set of features derives from the co-occurrence matrix, a  $6 \times 6$  table producing 36 karyometric features. The rows and columns of the table are defined by OD ranges for pixel A and B, respectively, in which pixel A is a given pixel under consideration, and pixel B immediately follows when the nucleus is scanned. Each feature captures the relative frequency of co-occurrence in the nucleus for a particular pixel–pixel transition. A fourth major set of features captures run lengths at varying OD intervals. Together, the third and fourth sets of features capture information about the heterogeneity of the chromatin distribution. A fifth set of miscellaneous features assessing measures such as entropy level and condensation value completes the overall feature set. A simple analogy to describe the more complex features would be to relate the scanned nucleus to a checkerboard with varying shades of gray. The relative frequency of co-occurrence would define how many different shades are in the checkerboard and the run length would indicate the size of a square for a given shade. The information contained in these 93 features allows detection of nuclear changes that reliably map onto progression curves for a wide variety of cancers. Discriminant function analysis using appropriate karyometric features more sensitively reveals steadily increasing deviations from normal tissue as pathologic gradation worsens.

### ***Applied Karyometric Analyses***

Applied karyometric analyses have been successfully used on diverse organ sites including skin, cervix, colon, epithelium, ovary, breast, bladder, and prostate. Differences have been shown not only between normal-appearing tissue and frank lesions, but also between normal-appearing tissue from lesion-free individuals and normal-appearing tissue from patients with a distant lesion. Such capability suggests that early detection may be possible in biopsies even when the lesion itself is missed by the sampling procedure.

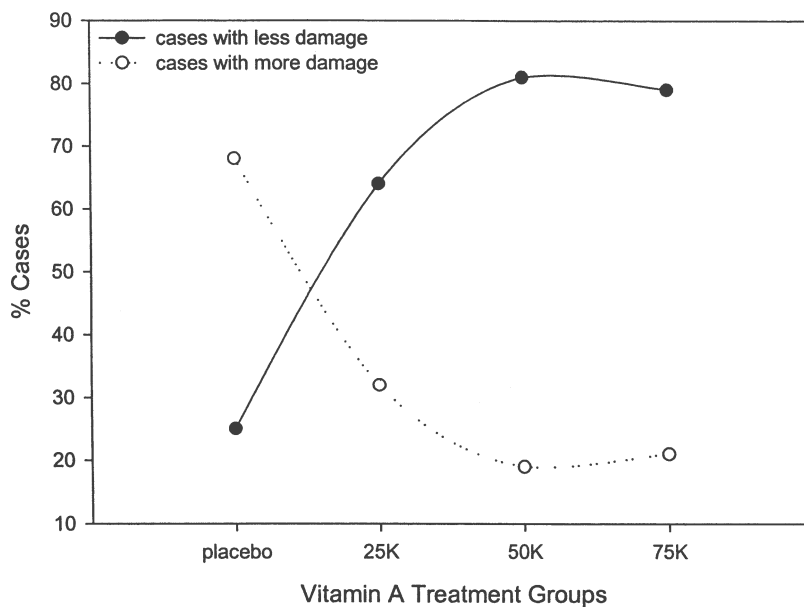


Fig. 6. Karyometric analyses of treatment of skin carcinogenesis with vitamin A. Analysis of the chemopreventative efficacy of daily oral vitamin A for 1 yr in skin for 113 subjects showed a dose response between vitamin A and the percentage of cases showing increases or reductions in nuclear damage levels as measured by average discriminant function scores.

Karyometric analysis is also a promising approach for assessing efficacy of chemopreventive agents because it is extremely sensitive to early nuclear changes, can be used to assess reversibility of nuclear damage in response to interventions targeted to any stage of carcinogenesis, provides a means for assessing the statistical significance of such changes, and is relatively nonspecific with respect to particular pathways for carcinogenesis. In practical terms, the combination of high sensitivity with lower specificity suggests that this method is appropriate for assessing efficacy of both targeted agents and those agents whose mechanism of action is as yet unknown.

Karyometric features and the functional scores derived from them can be considered as surrogate end point biomarkers. As such, it is important to establish their validity. Factorial experiments are establishing the reliability of the method with respect to key steps in the preparation of the optical image, including staining, image acquisition, nuclear selection, and nuclear segmentation. Results show a very high degree of reliability.

Evidence for external validation is accumulating. A karyometric analysis of the chemopreventive efficacy of vitamin A in skin showed a dose response between vitamin A and the percentage of cases showing increases or reductions in nuclear damage levels as measured by average discriminant function scores. A total of 22,600 nuclei were recorded from 113 cases at baseline and after 1 yr of treatment (Fig. 6) (89). This ability of karyometric analysis to detect a chemopreventive effect of vitamin A is validated by a larger clinical trial ( $n = 2297$  subjects), with an end point of the incidence of

SCC. In this study, vitamin A supplementation for up to 5 yr reduced the hazard ratio for first new SCC by 26% (204,441). Thus, the results found by karyometric analysis are consistent with cancer end point outcomes. The evidence to date suggests great promise for the karyometric methods described here as methods for early detection of progression to cancer and for assessment of the efficacy of chemopreventive agents and agents targeted at the critical stages of carcinogenesis.

## **Analysis of Gene Expression: Microarray**

### **Background**

Accurate diagnosis and effective treatment of cancer rely on our ability to recognize a recurring repertoire of clinical signs and symptoms that allow meaningful classification. In many instances, clinical signs and symptoms are poor predictors of clinical outcome or response to therapy. Because gene expression determines biologic phenotype, gene expression analysis, in the form of transcript profiling of tumor types representing various stages of disease and correlation with clinical end points, may provide insights into specific disease mechanisms and identify novel targets for therapeutic intervention. Useful diagnostic and prognostic markers may also be rapidly identified using transcript profiling.

Because thousands of transcripts are simultaneously and quantitatively analyzed on microarrays, this technology can provide a snapshot of gene expression encompassing large portions of and, at some point, the entire genome. This technology was not previously possible. Molecular phenotyping of tumor types through transcript profiling may therefore provide diagnostic, prognostic, and mechanistic insights that will improve the management of cancer. Microarray analysis has had an impact in basic science laboratories, providing an efficient way to globally assess the transcriptional effects of specific genetic or pharmacologic interventions, thus rapidly identifying possible downstream effectors or interacting pathways. In this context, the sheer number of transcripts screened in an unbiased fashion is a great strength of this approach, substantially enhancing the likelihood of discovering biologically important and previously unappreciated connections (442,443).

Arrays containing multiple DNA sequences arranged in a matrix were first developed during the early phase of the Human Genome Project to aid in the automation of DNA sequencing. Not long after that, photolithographic techniques enabled Affymetrix (Santa Clara, CA) to build the first high-density DNA array in 1994 (444,445). Smaller arrays containing ever-increasing numbers of DNA sequences called DNA microarrays or chips evolved from these early efforts. DNA microarrays have been used to quantify mRNA expression or to determine single-nucleotide polymorphisms.

Most microarrays consist of a solid support, usually a glass slide or nylon membrane, onto which DNA sequences are attached. DNA is either spotted, using pins or an ink-jet printer, or synthesized directly on the array using polymerase chain reaction or photolithography. The DNA may be either double-stranded copies of transcripts or shorter single-stranded oligonucleotides. For microarray analysis, RNA is first extracted from a sample. If the RNA yield is small, expressed sequences may be amplified. Although linear amplification of reasonable fidelity is generally possible, it is important to remember that this could introduce artifactual distortion of the original expression pattern. Subsequently, the RNA itself, complementary DNA, or amplified RNA is labeled using fluorescence or radioactivity. The labeled probe is hybridized, competitively or noncompetitively, to the microarray. Complementary sequences remain bound to the array and unbound sequences are washed off. Expressed genes are identified by the position of bound probes on the array.

### **Clinical Application of Microarray**

Growing evidence from small clinical studies suggests that valuable insights into disease classification can be obtained from transcript profiling. For example, microarray analysis of histopathologically similar breast tumors identifies expression patterns that persist in individual patients (446–449). Transcript profiling of histopathologically indistinguishable B-cell lymphomas revealed molecularly distinct disease subtypes that were associated with different prognoses and responses to treatment (450–452). Similarly, expression profiling of renal cell carcinoma enhanced prognostication compared with standard criteria alone (453). Microarray analysis also identified previously unappreciated subtypes of human melanoma and a novel subset of genes involved in their malignant transformation. Inhibition of one of these genes, *RhoC*, reduced metastasis in an animal model (454,455). Thus, transcript profiling can provide not only useful clinical markers but also mechanistic insights and potential targets for intervention at different stages of carcinogenesis.

### **Treatment of Carcinogenesis Related to Bacterial Infection**

Bacterial infections traditionally have not been considered to be major causes of cancer; however, over the last several decades, bacteria have been linked to several cancers and may act by several mechanisms including induction of chronic inflammation and production of carcinogenic bacterial metabolites and expression of viral oncogenes in tissues of the host. The most well-characterized examples of carcinogenesis directly linked to bacterial infection is association of gastric carcinoma and *H. pylori* infection.

#### **Gastric Cancer**

Gastric cancer is the second most common cause of cancer-related death worldwide (456). *H. pylori* infection has been associated with the development of gastric cancer and is classified as a group I carcinogen (457,458). Seroepidemiologic studies have associated *H. pylori* infection and an increased risk for the development of gastric cancer. The pathogenesis of *H. pylori* and its induction of cancer is unknown; however, all individuals infected with *H. pylori* do not develop cancer.

Gastric cancer has been described as a multifactorial and multistep process beginning with gastritis, followed by atrophic gastritis, intestinal metaplasia, dysplasia, and ultimately carcinoma (459). Nutrition, environment, and *H. pylori* infection can influence the development of gastric cancer (460,461). A major risk factor for the development of gastric cancer is chronic inflammation (containing infiltrates of lymphocytes and inflammatory cells such as neutrophils) of the stomach resulting in tissue damage (462,463). The onset of gastric carcinogenesis may be linked to damaging effects of the immune system in an effort to rid *H. pylori* from the body (464).

*H. pylori* infection in Mongolian gerbils induces chronic gastritis, ulcers, and intestinal metaplasia; the development of gastric cancer was observed in 37% of animals, indicating the involvement of *H. pylori* and cancer initiation. Therefore, modalities to eliminate *H. pylori* to thwart the onset of gastric cancer may decrease the incidence of gastric cancer. Numerous intervention studies have been completed in which *H. pylori* was eradicated (reviewed in ref. 456). Clinical studies indicate that *H. pylori* eradication is beneficial in preventing a malignant phenotype (459,465–468).

### ***Treatment of Carcinogenesis Related to Viral Infection***

Viral infection has a role in the pathogenesis of specific cancers; however, the incidence of such cancers is much lower than the frequency of virus infection, suggesting either that infection alone does not result in cancer and that cellular events in addition to the presence of the virus must occur, or that cancer occurs only if viral proteins are expressed in an appropriate cell type or in an immunocompromised host. Research analyzing virally mediated carcinogenesis has revealed that function, in part, by encoding proteins that inhibit the function of tumor suppressor proteins expressed by the host cells. Better understanding of the mechanisms underlying this association could have a profound effect on treatment of the associated cancers in the early stages of carcinogenesis. Knowledge of the transmission of cancer-associated viruses provides an opportunity for cancer prevention by enabling vaccination against the respective virus.

### ***Cervical Cancer***

Cervical cancer is the second most common cancer among women (469). HPV is thought to be the primary carcinogenic factor of cervical cancer and can be detected in >90% of squamous cell cervical cancers (470). However, HPV infection alone is unable to cause transformation of cervical cells; further genetic alterations are necessary to observe malignant transformation. Prevention of HPV infection or the eradication of established infection will be beneficial in preventing the onset of HPV-related cervical cancer.

More than 100 HPV genotypes have been discovered (471,472) and, of these, 35 infect the genital tract (473). HPV 16, 18, 45, and 56 are associated with a high risk of cervical cancer (474,475). The most prevalent cancer-inducing genotype is infection with HPV 16, resulting in invasive squamous neoplasia. Both therapeutic and prophylactic vaccines are being tested in preclinical and clinical studies. The E6 and E7 HPV proteins are involved in the disruption of cell-cycle regulation, resulting in progression from the G1- to S-phase. Additionally, E6 inactivates p53, a well-studied tumor suppressor. E6 and E7 proteins are constitutively expressed by tumor cells, making them ideal targets for immunotherapy (476).

HPV prophylactic vaccines activate the humoral immune response to generate neutralizing Abs that will bind to the virus before cellular infection. By coating the virus with such Abs, phagocytic immune cells will be more likely to detect and eliminate the pathogen, a process known as opsonification. Other HPV strategies include the development of peptide, viral-like particles and DNA vaccines to stimulate the cellular components of the immune response, specifically Ag-specific T cells (477–479).

### ***Hepatocellular Carcinoma***

A 100% seropositivity rate for hepatitis B surface antigen (HBsAg) was detected in Taiwanese children diagnosed with hepatocellular carcinoma (480,481). In 1984, Taiwan began a nationwide HBV vaccination program (482). Children were vaccinated and further monitored for the frequency of hepatocellular carcinoma. The incidence of hepatocellular carcinoma decreased significantly from 0.7 (1981–1986) to 0.36 (1990–1994) per 100,000 children age 6–14 yr. In children age 6–9 yr, the rate of cancer declined from 0.52 (1974–1984) to 0.13 (1984–1986) per 100,000 children. These data indicate that the vaccination program was effective at controlling HBV infection and preventing hepatocellular carcinoma. While liver cancer occurs primarily in adults

**Table 3**  
**Potential Agents For Treatment of IEN**

Agent	Class and source
Celecoxib	} COX-2 inhibitorsynthesized product
Rofecoxib	
Aspirin	} COX-1/-2 inhibitorsynthesized product
Sulindac	
EGCG	Natural product in green tea and black tea
DFMO	Fluoromethylornithine - Polyamine synthesis regulation
Resveratrol	Natural product in grapes
Lycopene	Natural product in tomatoes
Vitamin D	Vitamin product in milk
Vitamin E	Vitamin found in vegetables
Selenium	Trace mineral in food sources
Retinol	Vitamin found in vegetables
Limonene	Natural product in lemon peel
Kalikrein	Natural product in green vegetables
Genistein	Natural product in legumes
Curcumin	Natural product in curries

between age 40 and 60 yr, the success of Taiwan's vaccination program suggests that vaccination for those at risk of HBV infection can aid in the prevention of HBV-induced carcinomas. Continued monitoring of vaccinated children into adulthood is a necessity to determine the efficacy of the vaccination program.

### ***Other Potential Agents for Treatment of IEN***

Chemoprevention and the treatment of carcinogenesis can be defined as the use of one or more agents to prevent or reverse the process of cancer development. Although progress has been made in identifying specific mechanisms that regulate carcinogenesis, further advances are needed to identify molecular and cellular targets for development and use of effective agents. Numerous compounds have been identified as potential chemopreventive agents. The safety and efficacy of each agent must be investigated in preclinical and clinical studies. The process of carcinogenesis requires multiple steps that affect more than one gene. Many of these genes regulate important cellular functions, so they are prime targets for chemopreventive agents; this makes development of agents targeted specifically against abnormal cells challenging.

Identification of the molecular mechanisms in the various stages of carcinogenesis is the subject of intense study. These studies will facilitate identification and development of compounds that are safe and efficacious. Of particular interest are drugs and natural compounds that inhibit activity of the COX family of enzymes and certain dietary factors including green tea polyphenols and other natural antioxidants. A summary of agents that have shown promise in the treatment of IEN is provided in Table 3.

### ***d-Limonene and Monoterpenes***

Monoterpenes are nonnutritive dietary components found in the essential oils of citrus fruits and other plants. A number of these dietary monoterpenes have antitumor activity. These compounds have been shown to exert chemopreventive and chemothera-

peutic activities in several tumor models and represent a new class of cancer therapeutic agents. The naturally occurring monoterpene d-limonene, a major component of orange peel oil and the prototype monoterpene in carcinogenesis studies, is formed by the cyclization of the 10-carbon isoprene intermediate geranylpyrophosphate. d-Limonene inhibits various stages of tumorigenesis in several of animal models and is being evaluated as a chemotherapeutic agent in humans. Monoterpenes appear to act through multiple mechanisms in the chemoprevention and chemotherapy of cancer. The monoterpene limonene and perillyl alcohol are in clinical evaluation in cancer patients. d-Limonene is well tolerated in cancer patients at doses that may have clinical activity.

The chemopreventive efficacy of limonene during both the initiation and promotion stages of carcinogenesis has been shown in chemically induced rodent mammary (484–487), skin (488), liver (489), lung and forestomach (490), colon (491), and gastric (492,493) tumor model systems. d-Limonene inhibits activation of tobacco-specific carcinogens and accordingly may have the capacity to diminish carcinogenic response to exposures to tobacco (493). Dietary d-limonene has therapeutic effects against chemically induced mammary tumors in rats, with regression of >80% of carcinomas with little host toxicity.

A 10% limonene dose mixed in the diet caused tumor regression in all animals (494). The limonene dose-tumor regression response relationship was steep. While most tumors completely regressed with a 7.5% dietary level, significant regressions were not observed at 5% dietary levels. d-Limonene appeared to act in a cytostatic fashion. Its removal from the diet resulted in a significant number of tumor recurrences (495).

The effects of prolonged oral administration of d-limonene on gastric carcinogenesis induced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine and on the labeling and apoptotic indices of gastric cancers were investigated in Wistar rats. The data showed that d-limonene inhibited the development of gastric cancers through increased apoptosis and decreased DNA synthesis of gastric cancers (492). In another study, d-limonene attenuated the gastric carcinogenesis enhanced by sodium chloride via increased apoptosis and decreased incidence of gastric cancers in Wistar rats (496).

Chemotherapeutic activities of pharmaceutical preparations of d-limonene are under evaluation in phase 1 clinical trials (497). d-Limonene, as a drug, is well tolerated in cancer patients at doses that may have clinical activity. One partial response in a breast cancer patient on 8 g/m<sup>2</sup> per d (approx 14 g/d) was maintained for 11 mo, and three additional patients with colorectal carcinoma showed stabilization of disease for >6 mo on d-limonene at 0.5 or 1 g/m<sup>2</sup> per d (497).

The mechanisms by which d-limonene and other cyclic monoterpenes inhibit tumor growth have not been firmly established. Geranylpyrophosphate, the isoprene intermediate from which these compounds are derived, is required for synthesis of cholesterol, coenzyme Q (ubiquinone), and substrates used in the isoprenylation of several cellular proteins (498). d-Limonene inhibited carcinogen-induced neoplasia when administered at a short time interval before carcinogen challenge (486). The initiation-phase chemopreventive effects of d-limonene have been attributed to the induction of phase 1 (499) and phase 2 (500) carcinogen-metabolizing enzymes, resulting in carcinogen detoxification (499).

The postinitiation chemopreventive/tumor suppressive activity may be due in part to the inhibition of isoprenylation of cell growth-associated small G-proteins such as p21 ras by limonene and its metabolites (495,501). Perillic acid and dihydroperillic acid, the two major metabolites of limonene in the rat and human, are more potent inhibitors



of small-G-protein isoprenylation than is d-limonene. Posttranslational isoprenylation is required for functionality of these proteins, e.g., transformation by Ras (502,503). These findings, coupled with the extensive metabolism of d-limonene in vivo (504,505), raise the possibility that the antitumor effects of d-limonene in vivo may be mediated through perillic acid and other metabolites (504). Because farnesylation of ras protein is critical for its ability to cause oncogenic transformation, inhibition of protein prenylation may be the basis of the antitumor effects of d-limonene and perillic acid. A minor metabolite of d-limonene, perillic acid methyl ester, is a potent inhibitor of the enzyme protein farnesyl transferase. These data suggest that if the inhibition of protein prenylation is a mechanism for d-limonene's anticancer activities, this monoterpene may be a prodrug that is converted into pharmacologically active substances by metabolic modification. In a structure activity study, perillyl alcohol and other 7-mono-hydroxylated, d-limonene-derived monoterpenes were more potent inhibitors of protein isoprenylation and cell proliferation than d-limonene (506).

d-Limonene and d-limonene-related monoterpenes (perillyl alcohol and perillic acid) caused a dose-dependent inhibition of cell proliferation in breast cancer cell lines. The effects of d-limonene-related monoterpenes on cell proliferation and cell-cycle progression were preceded by a decrease in cyclin D1 mRNA levels (507). Moreover, d-limonene was shown to attenuate the gastric carcinogenesis enhanced by sodium chloride through increased apoptosis in gastric cancers (496). Another putative anti-tumor mechanism of action has been described for the monoterpenes. Tumors that regressed in response to d-limonene (508) and perillyl alcohol (509) had increased levels of TGF- $\beta$  protein and had increased expression of the mannose-6-phosphate/IGF type II receptor and TGF- $\beta$  type I, II, and III receptors at the protein and mRNA levels. The apoptotic index was increased approx 10-fold (510). These findings suggest that the monoterpenes inhibit tumor growth through a TGF- $\beta$ -mediated increase in apoptosis.

The chemopreventive effect of d-limonene against *N*-nitrosodiethylamine alone and with phenobarbital-induced hepatocarcinogenesis in AKR mice was studied. Over-expression of c-myc and c-jun at both mRNA and oncoprotein levels was completely inhibited when d-limonene was used along with *N*-nitrosodiethylamine or *N*-nitrosodiethylamine-phenobarbital. Thus, this study explained the antitumor effect of d-limonene, for the first time, on the level of oncogene expression in *N*-nitrosodiethylamine-mediated hepatocarcinogenesis (489). Perillic acid, a major metabolite of d-limonene, substantially suppressed IL-2 and IL-10 production in mitogen-activated T-lymphocytes. Perillic acid was shown to interrupt signaling via the Ras/MAPK pathway by depleting farnesylated Ras levels, an effect that may contribute to its inhibition of IL-2 production and T-cell activation (511).

### **Resveratrol**

The best candidates for successful chemopreventive agents are designed to elicit inhibitory properties at more than one stage of tumorigenesis. Preliminary studies have shown that resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a natural product found in grapes, berries, and other plants, may have potential as a cancer chemopreventive agent (512–516). Resveratrol was first shown to act as an antioxidant and antimutagenic agent, acting as an anti-initiation, antipromotion, and antiprogession agent (517–519). Further evidence indicated that resveratrol selectively blocks transcription of the phase I metabolic enzyme cytochrome P-450 1A1, which is involved in formation of DNA adducts in models of chemical carcinogenesis (520). In an in vitro model of mouse

mammary carcinogenesis, resveratrol was shown to inhibit the formation of carcinogen-induced preneoplastic lesions (521,522). Treatment of primary cultures of human mammary epithelial cells with 50  $\mu$ M resveratrol led to a decrease in the formation of DNA adducts ranging from 31 to 69%. Resveratrol also suppressed activity of phase II metabolic enzymes associated with chemical carcinogenesis, *O*-acetyltransferase and sulfotransferase in a mouse mammary carcinogenesis.

Resveratrol inhibits the formation of TPA-promoted mouse skin tumors in the two-stage model of carcinogenesis. In additional in vitro studies in cell-free models, resveratrol was shown to inhibit the enzymatic activities of COX-1 and -2 and COX-2 transcription of the *COX-2* gene (521,522). COX-1 and -2 activities were shown to be inhibited by resveratrol in a rat model of esophageal cancer. TPA-induced activation of protein kinase C and AP-1-mediated gene expression was blocked by resveratrol in mammary epithelial cells. In addition, resveratrol strongly inhibits nitric oxide generation and inducible nitric oxide synthase protein expression. Regulation of target genes by resveratrol may be largely due to inhibition of transactivation activity of the transcription factor NF- $\kappa$ B.

### Curcumin

Curcumin, also known as diferuloyl methane, is a yellow pigment in the spice turmeric, extracted from the herb *Curcuma longa* linn. Its properties as a colorant and flavor have led to its use in a variety of foods including saffron, curries, soups, and meats (523–526). Curcumin extracts have been used for medicinal purposes in India and Southeast Asia for generations in treatments for a variety of conditions, including inflammation, skin wounds, hepatic dysfunction, and certain types of cancers. In addition, curcumin has shown anticarcinogenic activity in animals, as indicated by its ability to block colon tumor initiation by azoxymethane and skin tumor promotion induced by the phorbol ester TPA (527,528). Curcumin has shown promise in vitro as a chemopreventive agent for tumors of the colon (529,530), prostate (531,532), and breast (533,534).

### Conclusion

The process of carcinogenesis can take decades, allowing multiple points at which intervention could be successful. Research on the paradigm of chemoprevention focuses on treatment of early stage carcinogenesis defined as IEN. As shown in Fig. 1, innumerable opportunities to intervene can be found along the pathway of carcinogenesis. Despite research and development efforts, indications for chemoprevention have been slow to emerge due to several factors, including the challenges in identification of appropriate populations and characterization of agents with acceptable toxicity profiles. Furthermore, because the ultimate end points in chemoprevention studies are development of cancer and cancer morbidity and mortality, long-term follow-up is necessary, which significantly increases costs.

Characterization of the molecular mechanisms associated with the process of carcinogenesis and identification of precursor lesions are the subject of intense study in the field of cancer research. Very strong evidence exists that patients diagnosed with IEN are at significantly higher risk of developing carcinoma. Although not all patients with IEN will develop cancer, screening and treatment of IEN may provide profound clinical and economic benefit. Clearly, development of novel agents is an appropriate goal for treatment of carcinogenesis.

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# 20

## Suicide Gene Therapy

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### Introduction

The most outstanding progress in molecular biology during the twentieth century led from the understanding of the genetic code, to the development of DNA technology, which led ultimately to gene therapy. These advances raised hopes that cancer could be cured using such an approach. The area of cancer gene therapy is vast and targets both malignant and nonmalignant cells for therapeutic gain. Gene therapy that targets malignant cells embraces a large spectrum of methods including the insertion of tumor suppressor genes (TSGs), cytokine genes, toxin genes, and prodrug-activating genes. This chapter deals with the latter, also termed *suicide gene therapy*. Basically, this approach uses DNA technology to transduce in cancer cells a gene able to activate a nontoxic prodrug into a cytotoxic drug able to kill the cancer cell population. This area of research is considered one of the most popular because this technique is represented by 52 clinical protocols (10.4%) including a total of 567 patients (16.5%) in 2001; some protocols are reported as combination suicide gene therapy (83 protocols).

Suicide gene therapy, also termed gene-directed enzyme prodrug therapy (GDEPT), is a way to improve cancer chemotherapy by selectively activating prodrug in tumors. The gene expressing the enzyme is transduced into the cancer cell using a vector or vehicle. The biggest challenge for GDEPT remains selective gene delivery to malignant cells.

### Suicide Gene Therapy: Background

The possibility of rendering cancer cells more sensitive to drugs or toxins by introducing suicide genes has two alternatives: toxin gene therapy, in which the genes for toxic products are transduced into tumor cells, and enzyme-activating prodrug therapy, in which the transgenes encode enzymes that activate specific prodrugs to create toxic metabolites. The latter approach, as well as suicide gene therapy and GDEPT (1,2) has also been termed *virus-directed enzyme prodrug therapy* (3), and *gene prodrug activation therapy* (4).

GDEPT is a two-step treatment designed to treat solid tumors. In the first step, the gene for a foreign enzyme is targeted in a variety of ways to the tumor where it is to be expressed. After expression of the foreign gene at the tumor, a prodrug is administered

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in the second step. Ideally, the gene for the enzyme should be expressed exclusively in the tumor cells and not in normal tissues and body fluids. The enzyme must reach a concentration sufficient to activate prodrug for clinical benefit. The catalytic activity of the expressed protein must be adequate to activate the prodrug under physiologic conditions. Since expression of the foreign enzymes will not occur in all cells of a targeted tumor in vivo, a bystander effect is required, whereby the prodrug is cleaved to an active drug that kills not only the tumor cells in which it is formed, but also neighboring tumor cells that do not express the foreign enzyme (5). The main advantages of suicide gene therapy systems are as follows:

- Increased selectivity for cancer cells with reduced side effects
- An amplification effect as one molecule of enzyme can activate many prodrug molecules
- Higher concentrations of active drug at the tumor compared with the concentrations permissible by classic chemotherapy
- Generation of bystander effect
- Induction of a beneficial immune response by tumor cell enzyme transduction and kill.

## Vectors in Suicide Gene Therapy

Suicide gene therapy requires vectors or vehicles capable of efficient and selective delivery of the therapeutic genes to tumor cells. For applications such as ex vivo infections, direct administration of vector to target tissues in vivo, or locoregional delivery, the ability to target specific cells may not be necessary. If systemic delivery is required, however, targeting is of major importance.

A number of vector systems have been proposed for gene therapy. These include viral vectors (adenoviruses [Ads] [6], adenoassociated viruses [7]; herpes simplex virus [HSV] [8], parvoviruses; lentiviruses, retroviruses), and nonviral vectors (naked DNA [with or without electroporation] [9], bacteria, cationic lipids, liposomes, polyamino acids, peptides, dendrimers) (10–12). The issues of major importance are the targeting, efficiency of infection or transfection, and safety of administration in humans.

Ads have achieved better infection rates (10–50%) in vivo than retroviruses (0.9–14.6%). Nonviral vectors with electroporation have achieved up to 8% transfection in vivo. Unusually high values (up to 59%) have been reported for nonviral vector transfection in vivo; however, the highest values (>80%) were reported for a combination of viral and nonviral vectors (Ads complexed with chemotherapy with polyethyleneimines [PEI] or diethylaminoethyl-dextran).

## Nonreplicating Viral Vectors

One concern of gene therapy is related to the hazards associated with the administration of viral vectors in humans. One way to reduce the risk is to use nonreplicating Ads and retroviruses. This category of vectors presents several advantages, especially in terms of transduction efficiencies. Unfortunately, gene therapy trials have shown that gene transfer remains disappointingly low, with primary tumors proving more resistant than animal xenograft models (13). Nontumor cells may become infected during the transfection procedures. Both conceptual and practical progress, however, has recently been made to overcome these deficiencies.

The lack of coxsackie adenovirus receptors (CARs) in many primary tumor cells was identified as the main cause of their resistance to transfection with adenoviral vectors. For example, CAR deficiency seems to be a near-universal feature of epithelial neoplasms (13). To date, Ads have been used widely because they can be produced

in high titers, can infect many different cell types, and can produce a transient expression of the transgene. This knowledge led to the development of adenoviral vectors capable of 'CAR-independent delivery' by retargeting to alternative receptors selectively expressed on tumor cells.

The strategies used for the design of CAR-independent Ads were reviewed (14). One strategy is based on retargeting using antiadenoviral fiber antibodies (Abs). A typical example is the development of the EpCAM-targeted adenoviral vector. EpCAM is a surface antigen that is overexpressed in most adenocarcinomas compared with normal epithelial counterparts. The vector was targeted to EpCAM through a bispecific Ab conjugate antiEpCAM/antiknob. Targeting ratios between 0.6 and 5.9 were achieved on clinical samples of gastric and esophageal cancers (15). Using a similar strategy, retargeting to other cellular receptors including integrins, the  $\alpha$ -folate receptor, fibroblast growth factor receptor (16) and epidermal growth factor receptor has been achieved. A potential disadvantage of this approach is the uncertain stability of the virus conjugates as well as the complexity of the system.

An alternative strategy uses a genetically modified targeted viral particle. Insertion of an RGD motif into the HI-loop of the adenoviral fiber knob resulted in efficient CAR-independent vectors by promoting the binding of the virus to integrins (17). The resulting vector, Ad-luc-RGD, containing a recombinant fiber RGD protein and expressing luciferase, revealed efficient CAR-independent infection of pancreatic carcinoma cells (17).

A procedure involving the combination of a retrovirus and an Ad expressing the same therapeutic gene was suggested. In vivo, this dual system was 21.5% more efficient than either adenoviral or retroviral delivery alone (18). Finally, it was shown that the CAR concentration on the surface of the cancer cells can be pharmacologically manipulated. The deacetylase inhibitor FR901228, sodium butyrate, and trichostatin A are able to increase CAR concentration. Cells treated with FR901228 before infection showed a 4- to 10-fold increase in transgene expression from a  $\beta$ -gal adenoviral vector (19).

### **Replication-Selective Viruses**

A different way to enhance gene transfer is to use posttransductional amplification by replicating viral vectors (5,13,20,21). A variety of viruses have been used, including Ads (22) and herpes viruses (23). An antitumor effect can be achieved directly by the conditionally replicative viruses or oncolytic viruses. These vectors spread and proliferate within the tumor and the transgene may be extended both temporally and anatomically. Another advantage of this approach is that the replicating viruses can deliver therapeutic transgenes called armed replicating viruses, thus enhancing potential for the eradication of the tumor (5,20).

In a study using an HSV-replication-competent vector containing LacZ as marker gene, a transduction efficiency in vivo in U87D glioma cells of 40% was demonstrated compared with 10% for the nonreplicative vector counterpart. LacZ transfection was stable for 14 d for the replicative vector, whereas it decreased sharply after 3 d for the nonreplicative vector (24). Recombinant HSV replicative virus is capable of infecting many types of cells, allowing insertion of large DNA sequences or multiple genes. Antiviral drugs are capable of controlling the infection. G-207 is a second-generation, conditionally replicating recombinant HSV-1 virus designed for clinical use in malignant brain tumors. The vector also had a LacZ gene inserted and harbored an intact *TK*

gene. Gene therapy using this vector followed by the prodrug ganciclovir (GCV) generated conflicting results, despite the high sensitivity of the transfected cells to the drug. GCV may induce the death of the G-207-infected cells and generate a bystander effect. Furthermore, GCV-mediated cell death may elicit a favorable immune response against tumor cells, thus enhancing the overall antitumor effect. On the other hand, GVC can inhibit G-207 replication, thus preventing the spread of the virus and its oncolytic effect. Phosphorylated GCV can produce the premature termination of the replicating DNA strands and affect both viral and genomic DNA synthesis (23,25). These opposing mechanisms of action support the observation that treatment with GCV, after administration of the replication-competent HSV-1, does not enhance its antitumor effect in certain situations (25).

Introduction of the cytochrome-P450 CYP2B1 gene into an HSV-1 vector, however, showed that the prodrug cyclophosphamide had a minimal effect on the viral infection and enhanced the antitumor effect of the virus (5,26). Similar results were obtained with the HSV-1yCD vector in conjunction with 5-fluorocytosine treatment, where the antitumor effect was augmented and the viral replication was unaffected (27).

Replication-selective Ads have been engineered (20). The most well known is ONYX-015, which has a deletion in the Ad E1B region enabling it to replicate preferentially in tumor cells. Insertion of the *TK* gene into this vector (AdTK<sup>RC</sup>) followed by administration of GCV augmented the antitumor effect with respect to the virus alone in melanoma, cervical, and colon carcinoma xenografts (28,29). Later work, however, could not confirm the augmentation of the effect after GCV treatment (30–32). Although data suggesting that the addition of the *TK* gene in the replicating virus is not beneficial, the concept of ‘armed’ replicating viruses is likely to have benefits using other GDEPT systems.

### **Bacterial Vectors**

Bacterial vectors have been developed for use in gene therapy. One example, *Salmonella typhimurium*, localizes to tumors after systemic injection in mice. The wild-type pathogen led to death in the mice, whereas attenuated, hyperinvasive auxotrophic mutants (by deletion of the *mbH* leading to lipid A metabolism) showed specific melanoma targeting after iv administration, with tumor:liver ratios in the range of 250:1 to 9000:1. When inoculated into C57BL6 mice bearing B16F10 melanomas, tumor growth was suppressed and prolonged animal survival resulted. A *Salmonella* vector containing the *TK* gene under the control of a  $\beta$ -lactamase secretion signal was developed and after GCV treatment showed efficacy in in vivo systems (22,33). *Salmonella* expressing HSV1-TK was recently proposed as an imaging agent because [<sup>14</sup>C]FIAU accumulation was bacteria dependent in mouse tumors (34).

Other bacteria, such as *Clostridium acetobutylicum* and *Bifidobacterium longum*, selectively germinate and grow in hypoxic regions of tumors after iv injections. An accumulation tumor:liver ratio  $>10^3$  was reported for *Bifidobacterium* (35). *Bifidum* bacteria harboring marker genes were constructed, and this gene delivery system was claimed to be tumor specific and nontoxic.

Spores of the anaerobic, apathogenic bacteria *Clostridium* were shown to germinate and proliferate only in tumors. To obtain an efficient infiltration of *Clostridium* in the tumor, at least  $10^7$  spores had to be systemically administered. In tumors, stable concentrations of  $10^9$  colony-forming units/(CFU)/g were detected. In normal tissues the concentrations ranged between  $10^4$  and  $10^6$  CFU/g and decreased with time (36).

*Clostridium* was genetically engineered to express the tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and CD genes. The specificity of *Clostridia* was further improved by using a radiation-induced promoter to control the therapeutic genes (36,37). A recent report describes the use of the clostridial strain *C. sporogenes* for the expression of CD. This strain has the highest reported colonization efficiency and can be systemically injected as spores. Another advantage is that this mode of delivery does not elicit an immune response (3).

### **Nonviral and Viral/Nonviral Hybrid Vectors**

An alternative to viral vectors is the nonviral strategy (11,12). This includes transfection procedures such as injection of naked DNA, and the use of physical devices such as gene guns, jet injection, and electroporation (38,39). More common systems are based on noncovalent complexes of carrier molecules and plasmid DNA, however. Such systems are suitable for systemic gene delivery to tumors and/or metastases. The development of such carrier molecules is difficult because of the biologic barriers, which must be overcome. Major advantages are linked to reliability, safety, and the fact that large expression cassettes can be transferred by this procedure. Using plasmid in conjunction with electroporation, an in vivo transfection efficiency of 3–8% was obtained compared with 0.1% for the same plasmid without electroporation (40,41). However, the approach is plagued by low transfection efficiencies especially in vivo. Differences in gene expression between rodents and humans have been reported.

The main categories of nonviral vectors used in gene delivery are those forming complexes with DNA. These include lipoplexes (such as cationic lipids or cytofectins) and polyplexes (such as poly-L-lysine–PLL, polyethyleneimine–PEI, peptides, dextrans, and dendrimers). For the lipoplexes, transfection efficiencies in vitro in the range of 0.2–35% have been reported (12,42,43).

An exciting development has been the combination of viral and nonviral strategies. One possibility is to use a viral/nonviral hybrid vector. Accordingly, liposomes in conjunction with the hemagglutinating Japan virus (HVJ–liposomes) were constructed that showed low immunogenicity and good in vivo transfection ability. The in vivo transfection efficiency in male severe combined immunodeficiency (SCID) mice bearing hepatocellular carcinoma tumors (HuH7 cells) was  $19.7 \pm 6\%$  (44). The same system used to transfer the *CD* gene to nude mice bearing BXPC3 human pancreatic tumor xenografts showed a transfection efficiency of approx 30% at d 3 (using *LacZ* as the marker gene). However, at d 7, almost no positive  $\beta$ -galactosidase cells were found (45). After administration of repeated doses of 5-fluorocytosine (5-FC) and also HVJ-CD liposomes, tumor size was reduced by 72% at d 28.

Progress has been made in the design of anionic HVJ–liposomes. In contrast to cationic liposomes, which do not penetrate tissues because of their net positive charge and large size, the HVJ–AVE anionic liposomes can penetrate tissues and exhibit higher efficiency of transfection. HVJ–AVE anionic liposomes with the envelope that mimics the human immuno-deficiency virus have been constructed, and the *LacZ* gene was transfected by intrathecal administration to the central nervous systems of nonhuman primates. Transfection efficiencies of 29–59% in neurons were reported (46).

An alternative strategy uses polycations to increase the adenoviral-mediated expression of the transgenic protein. Complexation of Ads harboring the *LacZ* gene with PEI allows the selective transfection of biliary epithelia by biliary canulation. Administration in vivo of  $1 \times 10^9$  pfu of Ads cocomplexed with PEI led to >80% infected epithelial cells, whereas Ads alone infected <5% (47).

Finally, a variety of ligands have been examined for their liposome-targeting abilities, including folates and transferrins. Folate-containing cationic liposomes were optimized for the systemic delivery of *p53* gene to mice carrying JSQ-3 xenografts derived from tumors of the nasal vestibule, which had failed radiation therapy. Transfection efficiencies of 40–50% were achieved after systemic administration of the vector (48).

### **Targeting Cancer Cells**

The targeting of cancer cells is important for the success of suicide gene therapy. A number of targeting possibilities have been described, including structural changes of the viral envelopes that allow selective interactions with specific receptors overexpressed on the surface of the cancer cells (see Nonreplicating Viral Vectors) (49); specific transcriptional regulation using tissue-specific or inducible promoters, alternative splicing (50), translational control (51,52), and specific delivery strategies. The focus here is mainly on the transcriptional regulation using specific promoters.

#### **Promoter-Specific Expression**

One way to achieve targeting specificity toward cancer cells is the use of tissue-specific promoters. This procedure is also known as tissue-targeted expression (49,53). If a tumor cell overexpresses a particular protein because of increased specific transcriptional activity of its promoter (rather than gene duplication), and a therapeutic gene is inserted downstream of this promoter, then introduction of this DNA sequence into these tumor cells should allow specific expression of the gene. Normal tissue that is also transduced would express much lower levels of the gene product and express none in an ideal system. This methodology (transcriptional selectivity) does not enhance transfection efficiency, but it is able to increase the expression of a therapeutic gene in cancer cells and to prevent or minimize the expression of the same gene in normal (surrounding) cells.

A number of promoters have already been investigated with positive results, including  $\alpha$ -fetoprotein (AFP) promoter, to target hepatocellular carcinoma; prostate-specific antigen (PSA), to target prostate cancer; Willebrandt factor, to target endothelial cells; DF3 MUC-1 promoter, to target breast cancer cells; tyrosinase promoter, to target melanoma cells; JC virus promoter and myelin-based promoter, to target glioma cells; prs-9 promoter, to target rhabdomyosarcoma cells; c-erbB2 promoter, to target breast, pancreatic, and nonsmall cell lung cancer cells; and osteocalcin promoter, to target osteosarcoma (49).

Recently, the midkine promoter was suggested for the treatment of pediatric tumors (Wilms' tumor and neuroblastoma). Midkine is a newly identified heparin-binding growth factor that is transiently expressed in the early stages of retinoic acid-induced differentiation of embryonal carcinoma cells and is overexpressed in many human malignant tumors. A recombinant replication-defective Ad containing the *TK* gene under the control of the midkine promoter followed by GCV administration achieved high activity in Wilms' tumors (G-401) and neuroblastoma. In contrast with adenoviral vectors harboring the *TK* gene under the cytomegalovirus promoter control, this system did not produce any liver toxicity after administration of GCV (54). The promoter of vascular endothelial growth factor (VEGF) that is activated by hypoxia was found to be useful in killing highly metastatic Lewis lung carcinoma A11 cells under hypoxic conditions. A retroviral vector was constructed harboring the *HSV-TK* gene under the control of the VEGF promoter. (55).

Several examples showed that the use of enhancers in conjunction with specific promoters in viral constructs may be beneficial. Placing a 1455-bp PSA enhancer sequence upstream of either the PSA or the glandular kallikrein promoter (hKLK2) increased the expression of the marker gene in the PSA-positive prostate cancer cell line LNCaP by 20-fold. Tandem duplication of the PSA enhancer increased expression to 50-fold while retaining tissue specificity. Furthermore, expression of all enhancer constructs was increased 100-fold above basal levels when induced with dihydrotestosterone. Adenoviral vectors produced on this basis and harboring either the epidermal growth factor promoter or nitroreductase genes were evaluated in LNCaP cells, showing selective expression in PSA-positive cells (56). On a similar basis, a hypoxia-inducible enhancer (a fragment of a human VEGF containing a hypoxia-responsive element) was coupled to AFP promoter in a retroviral vector with an *HSV-TK* gene. After transfection into hepatoma cells and exposure to 1% O<sub>2</sub> and GCV, specific toxicity was reported (57).

The L-plastin promoter has been proposed for use in gene therapy. It belongs to a family of genes encoding actin-binding proteins. Infection of ovarian carcinoma cells (OvCar-5 and SK-OV-3) with the recombinant replication-defective Ads containing the *CD* gene under the control of the L-plastin promoter followed by 5-FC treatment proved to be effective both in vitro and in vivo (58). Telomerase-specific suicide gene therapy vectors expressing bacterial nitroreductase were designed using hTER and hTERT telomerase transcriptional regulatory sequences. These constructs were able to sensitize human cancer cells to CB1954 (59).

The latency-associated promoter (LAP) was proposed for sustaining long-term expression in neurons. However, in vivo data indicate that although the HSV-1-LAP vector can drive the expression of the *TK* gene in a variety of central nervous system neurons, there is a slow downregulation of the promoter (60).

The Myc-Max binding motif (which activates the transcription of an adjacent promoter) was proposed for the treatment of small-cell lung cancer, which overexpressed *myc* family oncogenes (61). A Cre/loxP approach was suggested in conjunction with the CD/5-FC suicide gene therapy system for the treatment in vivo of gastric carcinoma models (62).

Finally, the osteocalcin promoter has been used in a conditionally replication-competent adenoviral vector. The recombinant Ad-OC-E1a vector harboring the osteocalcin promoter proved to be effective in inhibiting the growth of PSA-producing and -nonproducing human prostate cancer cell lines (63).

### Gene Regulation

In gene therapy, continuous production of a particular enzyme may be achieved after a single administration of vector. Continuous expression has its disadvantages, in some instances with strong viral promoters, leading to immunogenicity problems. Furthermore, there is no inherent control of the level of expression, which could easily stabilize outside the range desired for the therapy. It may be preferable to keep the levels and/or kinetics of expression within a controlled range. Toxins or powerful cytokines such as TNF- $\alpha$  are in frequent use in cancer gene therapy strategies. The safe use of such toxic agents requires some kind of quantitative control over their production especially in vivo.

Another problem associated with unregulated expression is encountered in the production process of gene therapy viral vectors. In many cases, these vectors harbor toxic genes such as TNF- $\alpha$ , or FasL; unregulated expression of such proteins may



give rise to producer cell-line toxicity. Because viruses need to be grown and amplified within cells such toxicity translates to poor or nonexistent viral titer yield (64). Furthermore, in the case in which a recombinant replicating or conditionally replicating viral vector is involved, one may wish to keep the inserted therapeutic gene under control because it may interfere with the viral replication cycle in vivo and reduce the efficacy of the treatment. Hence, it is essential to be able to maintain control of the expressed protein (64).

In the last decade, regulation has been imposed at different stages of gene expression. Transcriptional regulation has been the most common method investigated, although control at the level of translation, posttranslation, and secretion has begun to emerge.

Inducible promoters (by photodynamic therapy) belonging to the glucose regulated protein (grp) family were also used to control and enhance the therapeutic efficacy of the HSV-TK/GCV system (65). Another recent study shows that radiation-inducible promoters can induce the expression of TNF- $\alpha$  and CD in apathogenic *Clostridia* strains (37).

### **Current Enzyme/Prodrug Systems for GDEPT**

A system for suicide gene therapy is a two-part entity comprising an enzyme and the corresponding prodrug. In 2001, we published a table containing the main 20 GDEPT systems (66). Since its publication, some improved or new systems such as alkaline phosphatase/etoposide phosphate-releasing etoposide (50), hypoxanthine-guanine phosphoribosyl transferase/allopurinol (67), horseradish peroxidase/indole-3-acetic acid, tyrosinase/hydroxyphenylpropanol and *N*-acetyl-4-*S*-cysteaminyphenol (68), linamarase/linamarin-releasing hydrogen cyanide (69), and folylpolyglutamyl synthetase/edatrexate producing edatrexate-polyglutamate (26,70) have been described. The systems are in different stages of development, from in vitro preclinical studies (71) to phase 3 clinical trials (72).

### **Enzymes Used in Suicide Gene Therapy Systems**

There are specific requirements of the enzymes used in GDEPT, which should have high catalytic activity (preferably without the need for cofactors), should be different from any circulating endogenous enzymes, and should be expressed in cancer cells in sufficient concentration for therapeutic efficacy. The enzymes proposed for suicide gene therapy can be characterized into two major classes. The first class comprises enzymes of nonmammalian origin with or without human counterparts. Examples include viral TK, bacterial CD, bacterial carboxypeptidase G2, purine nucleotide phosphorylase, thymidine phosphorylase, nitroreductase, D-amino-acid oxidase, xanthine-guanine phosphoribosyl transferase, penicillin G amidase,  $\beta$ -lactamase, multiple drug activation enzyme,  $\beta$ -galactosidase, horseradish peroxidase, and deoxyribonucleotide kinase. Those enzymes that do have human homolog all have different structural requirements with respect to their substrates compared with the human counterparts. Their main drawback is that they are likely to be immunogenic. The second class of enzymes for suicide gene therapy comprises enzymes of human origin, which are absent from or are expressed only at low concentrations in tumor cells. Examples include deoxycytidine kinase, carboxypeptidase A, and cytochrome P450. The advantages of such systems reside in the reduction of the potential for inducing an immune response. Their presence in normal tissues is likely to preclude specific activation of the prodrugs only in tumors unless the transfected enzymes are modified for different substrate requirements.

The genes can be engineered to express their product either intracellularly or extracellularly in the recipient cells (2). The site of enzyme expression and therefore prodrug activation is an important factor in suicide gene therapy (73). The extracellularly expressed variants are either tethered to the outer cell membrane (74,75) or secreted from cells (74,76). Each approach has potential advantages. Where the enzyme is intracellularly expressed, the prodrug must enter the cells for activation, and subsequently the active drug must diffuse through the interstitium across the cell membrane to elicit a bystander effect. Cells in which the enzyme is expressed tethered to the outer surface or secreted are able to activate the prodrug extracellularly. A more substantial bystander effect could therefore be generated in the latter system, but spread of the active drug into the general circulation is a possible disadvantage (2,77).

Recently mutated enzymes were designed and used to improve the kinetics of activation of a certain substrate or to achieve uncommon structural requirements by the substrate to decrease the risk of interference by endogenous molecules.

### Prodrugs and Drugs for Suicide Gene Therapy

A prodrug designed for a GDEPT system should be a good substrate for the activating enzyme. Favorable characteristics of prodrugs for GDEPT include efficient prodrug activation even at low concentrations of prodrug (low  $K_m$ ) and rapid conversion of the prodrug to the active drug (high  $k_{cat}$ ) (71,78). The prodrug should not be a substrate for any endogenous enzyme, to avoid cytotoxic activation outside the tumor in normal tissues (78,79). Good physiologic stability of prodrug is required to prevent premature release of cytotoxic drug or, alternatively, deactivation of the prodrug before it reaches the expressed enzyme. A suitable pharmacokinetic profile in terms of bioavailability, biodistribution, area under the curve, and half-life in plasma is necessary. The cytotoxicity differential between prodrug and drug should be as high as possible to allow a comfortable therapeutic window. A minimum of 100-fold differential is considered by some researchers (71,78) to be necessary for significant therapeutic gains, although lower values have been reported to produce good biologic effect (77,80,81). With the emergence of replicatively competent viral vectors, bacterial vectors, and engineered macrophages, the interaction between prodrug and vector is a consideration. The prodrug should not release a drug that kills the transfection vector prematurely. On the other hand, prodrugs can be used to control the spread and adverse responses due to these new vectors (82,83).

The released drug should also fulfill several criteria. Ideally it should be active against both dividing and quiescent cells (78,79,84). Examples of drugs fulfilling this requirement are alkylating agents, 6-methylpurine, and 2-fluoroadenine (85,86). Its cytotoxicity should be as high as possible, to overcome potential limitations in prodrug penetration of tumors and in the capacity of the activation mechanism (87).

Bystander effect is compulsory for the drug, as only a percentage of tumor cells will be transfected or transduced and will express the activating enzyme. A highly diffusible drug is likely to mount a stronger effect. To diffuse freely in the interstitial space and cross the cells' membranes, the drug should ideally be a neutral, uncharged compound. This requirement does not apply if a different mechanism of effect or active transport of drug is involved. If a drug is too diffusible and stable, leakage into the general circulation will occur, with corresponding systemic toxicity. For this reason, the half-life of the drug should be optimized to achieve the right compromise between tumor diffusion and prevention of systemic escape (71,78,88).

Drugs that are acting directly without the requirement for extra endogenous enzymatic activation steps have an advantage in circumventing potential resistance due to low expression of endogenous enzyme. For most GDEPT systems, the enzyme is expressed intracellularly. Consequently, the prodrugs should be able to cross the cell membrane, either by passive diffusion or by active transport. If the expressed enzyme is tethered on the outer membrane of the cells (74,75,77) or secreted (74,76), this requirement is waived.

Two basic types of prodrugs have been used in GDEPT: the direct prodrugs and the self-immolative prodrugs (pro-prodrugs). The direct prodrugs can be defined as a pharmacologic inactive derivative of a drug, which requires chemical transformation to release the active drug. In terms of anticancer activity, the conversion of the prodrug to an active drug results in a sharp increase in its cytotoxicity. In a direct prodrug, the active drug is released directly after the activation process.

A self-immolative prodrug can be defined as a compound generating an unstable intermediate that, after the activation process, will extrude the active drug in several subsequent steps. The most important feature is that the site of activation is normally separated from the site of extrusion. The activation process remains an enzymatic one. The extrusion of the active drug relies on a supplementary spontaneous fragmentation. Potential advantages of self-immolative prodrugs are the possibility of altering the lipophilicity of the prodrugs with minimal effect on the activation kinetics and the possibility of improving unfavorable kinetics of activation due to unsuitable electronic or steric features of the active drug. The range of drugs that can be converted to prodrugs is greatly extended and is restricted only by the structural substrate requirements for a given enzyme.

Two ways of developing prodrugs for GDEPT have been investigated. The first is based on known prodrugs with various spectra of activity: antiviral (e.g., GCV), antibacterial (e.g., 6-methylpurine-2'-deoxyribonucleoside), antiparasitic (e.g., allopurinol), antifungal (e.g., 5-FC), antitumoral (e.g., 5'-deoxy-5-fluorouridine), or compounds that are known to produce toxic metabolites but are poorly or not at all activated in the tumors (e.g., selenomethionine [89,90], 2-aminoanthracene [91]). Transduction of the tumor with the corresponding activating enzyme achieves the tumor-selective cytotoxic effect.

The first generation of enzyme/prodrug systems employed well-known anticancer prodrugs of clinical use. The advantages were that their behavior, kinetics, and pharmacokinetics were already known and some of them are approved as drugs by the regulatory authorities. This status was useful for establishing the proof of principle that GDEPT systems were working.

The second route for the generation and development of enzyme/prodrug systems starts from enzymes with no prior prodrug substrate known. The specificity for the natural substrates and the structural requirements are well researched. The enzyme should catalyze a reaction with distinct substrate specificity but should allow modifications in certain parts of the substrate without large alteration of the activation kinetics. This site of accepted variability is exploited either to attach a known drug to the enzyme specifier or to modify the substrate in a convenient way such that it will generate a cytotoxic drug after enzymatic activation. Examples of enzymes for which prodrugs have been designed and synthesized for GDEPT include nitroreductase (92), carboxypeptidase G2 (93,94),  $\beta$ -glucuronidase (75),  $\beta$ -galactosidase (95), carboxypeptidase A (74), phosphatase (50), and tyrosinase (69).

Many prodrugs tailored for GDEPT attempt to take advantage of both types of approach: the prodrug is derived from a known antitumor drug, modified to become deactivated and a substrate for the foreign enzyme. Examples are anthracyclins, 5-fluorouracil (5-FU), methotrexate, and etoposide prodrugs. Other prodrugs have a more radical design: both the prodrug and the released drug are new entities with respect to clinical use. This category takes advantage of cytotoxic moieties that cannot be used as systemic drugs due to undesired side effects, but that become relatively nontoxic after suitable derivatization to prodrug. Activation of these prodrugs by the expressed enzyme releases the cytotoxic moiety locally, at the tumor site, minimizing side effects. Examples of this class are alkylating agents, pyrrolobenzodiazepines, enediyines, and amino-seco-cyclopropylindoles.

### **The Bystander Effect**

The bystander effect in a suicide therapy system can be defined as the cytotoxic effect on nongenetically modified cells after prodrug administration, when only a fraction of the tumor mass is genetically modified to express an activating enzyme (96). The successes described in GDEPT would not be possible without the existence of such an effect. Although the effect is difficult to quantitate especially *in vivo*, models have been devised to examine it. Other phenomena, such as the immune response, can contribute strongly to the overall effect.

### ***Mechanisms of the Bystander Effect***

The prodrugs activated in GDEPT systems can release active drugs that are either diffusible or nondiffusible across cell membranes. In the case of diffusible toxic metabolites formed after prodrug activation and released from dead and dying genetically modified cells, they will spread, according to diffusion laws, within the tumor cell population. This mechanism is postulated for most GDEPT systems such as 5-FU formed from 5-FC; for the metabolites of CP or IP, aldophosphamide, phosphoramidic mustards or acrolein; for benzoic acid mustard released from CMDA, and for 6-MeP, formed from the corresponding deoxynucleoside. The most relevant feature of such a mechanism is that cell-to-cell contact is not required for the killing of untransfected cells either *in vitro* or *in vivo*. The bystander effect is relying only on the diffusibility of the active drugs in the tumor interstitium and across the tumor-cell membranes and on their cytotoxic potential. A number of examples support this assumption.

For purine or pyrimidine nucleoside prodrugs, the toxic metabolites are generally phosphorylated and are not diffusible across cell membranes, so the mechanism of the bystander effect is quite different. The HSV-TK/GCV system requires cell-to-cell contact to display a bystander effect. The transfer of toxic metabolites from cell to cell mainly requires the existence of gap junctional intercellular communications (GJICs), but other mechanisms could be involved. The GJICs broadly vary among the types of cell lines and are measured using dye (e.g., Lucifer yellow) diffusion through gap junctions. The amount of GJIC is predictive of the extent of the bystander effect *in vitro*, whatever the origin of the cancer cell lines. Consistent with this model, a number of reports showed that tumor cells resistant to the bystander effect did not show dye transfer from cell to cell, whereas the bystander effect-sensitive tumor cells did (97). Some exceptions suggest that the bystander effect is not completely mediated by gap junctions, even if cell to cell contact is necessary (98). The bystander effect has been

observed *in vivo* and generally a relationship is found between *in vitro* and *in vivo* behavior. The bystander effect *in vivo* can be enhanced by collateral immune effects.

Another effect, the 'good Samaritan effect,' has been described. This effect refers to the observation that transfected cells can be protected from the active drug, presumably by lowering the concentration of the cytotoxic metabolites through GJIC (99,100). This is beneficial since the transgenes last longer, producing more toxic metabolites, thus enhancing the bystander effect. On the other hand, it can be regarded as detrimental, making the eradication of the whole cell population more difficult.

In the GDEPT systems involving diffusible metabolites, it is difficult to pinpoint methods to enhance the local transfer of the active drug. One possibility is to express the enzyme extracellularly on the surface of tumor cells. Another way is to release drugs, which can cross cell membrane by active transport.

Several options can improve the bystander effect based on the GJIC hypothesis for the GDEPT systems releasing nondiffusible toxic metabolites. GJIC can be controlled pharmacologically by using dieldrin, a drug that decreases cell-to-cell communications. The dye transfer is diminished, and dieldrin inhibited the bystander effect. Cyclic adenosine monophosphate (cAMP), forskolin, corticoids, carotenoids, and flavonoids (such as flavanone, apigenin, tangeretin) are able to induce GJIC *in vitro*. This effect may be cell specific or connexin specific. The bystander effect was induced *in vivo* with cAMP and retinoids (97). Apigenin and lovastatin, an inhibitor of HMG-CoA reductase, both upregulate gap junction function and dye transfer in tumors expressing gap junctions (101,102).

In one report studying human lung tumor cell lines of different origins, significant cell killing occurred when only 10% of cells expressed HSV-TK. In this system, GJICs were not apparent from measuring the rapid intercellular transport of Lucifer yellow, which detects 'rapid-transfer' gap junctional communications, although it could be seen by the slow transfer of a different dye, calcein-AM, which measures the slow-transfer gap junctions. Neither an inhibitor (1-octanol) nor an enhancer (all-*trans* retinoic acid) of gap junctions affected the extent of the bystander effect, suggesting either that low levels of gap junctions can produce a maximal bystander effect or that bystander cell killing occurs by other means (98).

GJICs are heavily dependent on the activity of connexins. The type of connexin expressed does not appear to be crucial for the bystander effect because similar results were obtained with cells expressing different types of connexins. It was, however, shown that the transfection of connexin genes into a number of different tumor cells (ie PC12 adrenal pheochromocytoma, HT-116 colon carcinoma, N2A neuroblastoma, and C6 glioma) significantly increased the bystander effect for the HSV-TK/GCV system. Transfection of *Cx43* gene into the MDA MB 435 breast cancer cell line restores GJIC and high expression of *Cx43* enhanced the bystander effect of the HSV-TK/GCV system. The noncommunicating MDA MB 435 breast cancer cell line triggered a significant bystander effect both *in vitro* and *in vivo* with the HSV-TK/GCV system, suggesting that mechanisms other than GJIC may be involved in the bystander effect (103).

Another explanation may be that the TK enzyme or the toxic metabolites can be transported by apoptotic vesicles in nontransfected cells. The fact that the bystander effect can be induced in the absence of apoptotic death in hepatocarcinoma cells and that the transfer of GCV-TP occurs before apoptotic degeneration opposes this assumption (103,104). Phagocytosis of material from dying TK-positive cells (e.g., hydrolases

or other lytic enzymes) to bystander cells has been suggested as a mechanism for the bystander effect. Apoptosis was detected in bystander cells and it was found that this event could be inhibited by *Bcl2* expression. During the apoptosis induction period, in bystander cells cocultured with HSV-TK-expressing cells, no phagocytosis was observed. It has been suggested that killing of tumor cells by apoptosis could heighten the immune response to wild-type tumor cells by a priming effect.

### **Immune Effects**

It is generally accepted that the immune response improves the efficacy of GDEPT systems in vivo. Several lines of evidence strengthen this view. The first line of evidence is that although the bystander effect has been observed in immunocompromised animals, a number of data suggest that the bystander effect in vivo is mediated largely through the release of cytokines (105) and, therefore, GDEPT systems are more efficient in immunocompetent animals.

The second line of evidence is the existence of the distant bystander effect. A distant bystander effect has been reported in a number of situations when tumors are anatomically separated with no possible metabolic cooperation and were both inhibited after suicide gene therapy was administered to only one tumor (4,106). An immune-related response has been proposed to explain this effect, but conflicting opinions have been noticed since previous reports described the occurrence of the distant bystander effect in immunodeficient animals. A new model was devised by implanting colorectal tumor cells in two lobes of the liver, followed by HSV-TK/GCV therapy to only one tumor. After administration of GCV, the distant tumors regressed partially or totally, the distant bystander effect being observed in 92% of animals. This study clearly demonstrated that the distant bystander effect was due to an immune response (106).

The third line of evidence is given by the cotransfection of both suicide genes and immune-enhancing genes. The transgenes containing both a suicide gene and *GM-CSF* or *IL* gene proved to be more effective when compared with the suicide gene therapy alone. An HSV-TK suicide gene therapy system in conjunction with *GM-CSF* gene was administered in Balb/c mice bearing M-26 colon carcinoma, followed by administration of GCV. Although no difference in the size of tumors compared with HSV-TK/GCV alone were seen, tumors regrew only in mice receiving the *TK* gene alone. Such combined systems are able to induce complete or partial resistance to a tumor rechallenge (107). Higher efficacy as well as antimetastatic activity was shown by the same HSV-TK+GM-CSF/GCV system in a model of metastatic breast cancer (108). Similar observations were reported for the CD/5-FC system. Intraperitoneal administration of AdSCF/AdGM-SCF in mice bearing CT26 colon adenocarcinoma followed by treatment with AdCF/5-FC could suppress tumor growth and prolong the survival period (109).

It was suggested that some drugs released during suicide-gene therapy in vivo could produce tumor necrosis and an inflammatory response, which may break down the immunologic isolation and elicit an immune response (110). Such drugs may have a definite advantage in comparison with those inducing apoptosis. It was believed that for the CYP2B1/IF system, the phosphoramidate mustard resulting after activation causes DNA crosslinks inducing cell death by apoptosis. A study demonstrated a necrotic mechanism of cell death, which may have important implications for the activation of the immune system (111).

## Clinical Evaluation

Gene therapy is a rapidly expanding area. Its progress can be measured in numbers of clinical trials generated since its beginning in 1989. In February 2001, 532 protocols including 3436 patients were reported from 359 phase 1 trials (67.5%) including 1774 patients, 57 phase 2 trials (10.7%) including 507 patients, and 3 phase 3 trials (0.06%) including 251 patients.

Clinical trials with ONYX-015 showed that this replicating viral vector is generally well tolerated at doses up to  $2 \times 10^{12}$  particles. Viral replication was tumor selective and transient ( $<10$  d) (112).

A number of clinical trials have been done with different suicide-gene therapies. An important consideration is the side effects of the different components, which may be elicited by the vectors, the enzyme, and/or the prodrugs. Clinical phase 1, phase 2, and phase 3 studies are ongoing with HSV-TK/GCV, CD/5-FC, CYP450-CP, and NR/CB1954.

## Conclusion

Major improvements are needed in vector design area to enhance targeting and delivery of suicide genes. Multiple options are available, including nonviral vectors, more complex systems involving coexpression of suicide genes with immunologic genes or TSGs, and selectively replicating viruses.

The combination of GDEPT with radiotherapy or immunotherapy has previously been investigated. Such approaches may involve either a sequential treatment schedule (GDEPT/radiation therapy or GDEPT/immunotherapy) or the transfection of suicide gene(s) together with genes able to increase the sensitivity of the tumors to radiation or enhance the potential of the host immune system with cytokine genes.

GDEPT systems have already shown efficacy *in vivo*. Future developments in this technology should use enzyme mutagenesis to obtain more efficient activation of a given prodrug, or to adapt the active site so that it binds better to prodrugs that are not substrates for endogenous enzymes. The prodrugs, too, could be redesigned to create better substrates for the enzymes, to maximize drug release or the bystander effect, to take advantage of self-immolative strategies of activation, or to allow the active drug to accumulate more readily in tumor cells.

The simultaneous release of active drugs that can act by different mechanisms, leading to a synergistic effect on tumor cells and the design of more effective new types of prodrugs, is another way to progress. Modalities to enhance and to control the bystander effect, particularly if cell-permeable and cell-impermeable active metabolites can be released together, may be useful to improve therapies. The occurrence of resistant populations is less likely for drugs with different mechanisms of action.

In conclusion, major improvements to the vectors and the enzyme/prodrug systems have already been made. The focus for the future is to combine these aspects into one effective therapy.

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## Glossary

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2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
5FU	5-fluorouracil
6DMAP	6-dimethylaminopurine
AAV	adeno-associated virus
Ab	antibody
ABC	ATP-binding cassette
ACS	American Cancer Society
Ad	adenovirus
ADCC	antibody-dependent cellular cytotoxicity
AF	activation factor
AFH	angiomatoid fibrous histiocytoma
AFP	$\alpha$ -fetoprotein
Ag	antigen
AgR	androgen receptor
AGP	acid glycoprotein
AGT	O <sup>6</sup> -alkylguanine DNA alkyltransferase
AHH	aryl hydrocarbon hydroxylase
AK	actinic keratosis
ALCL	anaplastic large cell lymphoma
ALL	acute lymphoblastic/lymphocytic leukemia
AML	acute myeloid/myelogenous leukemia
ANC	absolute neutrophil count
Ang	angiopoietin
AP	apurinic/apyrimidic
APC	anaphase-promoting complex <i>or</i> adenomatous polyposis coli <i>or</i> antigen-presenting cell
APE	apurinic endonuclease
APL	acute promyelocytic leukemia
AR	androgen receptor
ARF	alternative reading frame

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ASCO	American Society of Clinical Oncology
ASO	allele-specific oligodeoxynucleotide
AT	ataxia telangiectasia
ATBC	Alpha-tocopheral, Beta-carotene Cancer Prevention Trial
ATF	activating transcription factor
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad53-related
ATRA	all- <i>trans</i> retinoic acid
ATX	autotaxin
AUC	area under the curve
BAC	bacterial artificial chromosomes
BCC	basal cell carcinoma
BCG	bacillus Calmette-Guerin
B-CLL	B-cell chronic lymphocytic leukemia
BCR	B-cell receptor
BER	base-excision repair
BG	O <sup>6</sup> benzylguanine
bHLH	basic helix-loop-helix
BL	Burkitt's lymphoma
BMT	bone marrow transplantation
bp	base pair
BRCP	breast cancer resistance protein
C	constant
CAK	CDK-activating kinase
CaMKK	calcium/calmodulin-dependent kinase kinase
CAR	Coxsackie adenovirus receptor
CARD	caspase recruitment domain
CARET	Carotene and Retinol Efficacy Trial
CDC	complement-dependent cytotoxicity
CDGE	constant denaturant gel electrophoresis
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
CDR	complementarity-determining region
CEA	carcinoembryonic antigen
CFU	colony-forming unit
CFU-GM	granulocyte-macrophage colony-forming units
CGH	comparative genomic hybridization
CHO	Chinese hamster ovary
CHOP	chemotherapy with cyclophosphamide, doxorubicin, vincristine, and prednisone
CIS	carcinoma <i>in situ</i>
CISH	chromogenic <i>in situ</i> hybridization

CKI	cyclin-dependent kinase inhibitor
CLL	chronic lymphocytic leukemia
CMF	chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil
CML	chronic myelogenous leukemia
cMOAT	canicular multispecific organic anion transporter
CMV	cytomegalovirus
COX	cyclooxygenase
CRC	Cancer Research Campaign
CREB	cAMP-responsive binding protein
CSF	colony-stimulating factor
CT	computed tomography
CTL	cytotoxic T-lymphocyte
CTLA	cytotoxic T-lymphocyte antigen
D	diversity
Da	daltons
DAG	diacylglycerol
DBD	DNA-binding domain
DC	dendritic cell
DCC	deleted in colorectal cancer
DCIS	ductal carcinoma <i>in situ</i>
DEAE	diethylaminoethyl
DES	diethylstilbestrol
DFMO	difluoromethyl ornithine
DG-DGGE	double-gradient denaturing gradient gel electrophoresis
DGGE	denaturing gradient gel electrophoresis
DHAC	dihydro-5-azacytidine
DHFR	dihydrofolate reductase
DHPLC	denaturing high-pressure liquid chromatography
DIGE	differential gel electrophoresis
DLCL	diffuse large cell lymphoma
DLT	dose-limiting toxicity
DMBA	dimethylbenzanthracene
DPD	dihydropyrimidine dehydrogenase
DSB	double-strand break
Dvl	dishevelled protein
EBMT	European Bone and Marrow Transplant
EBV	Epstein-Barr virus
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELCAP	Early Lung Cancer Action Project



ELISA	enzyme-linked immunosorbent assay
EMC	extraskelatal myxoid chondrosarcoma
EMA	European Medicines Evaluation Agency
EORTC	European Organization for Research on the Treatment of Cancer
ER	estrogen receptor <i>or</i> endoplasmic reticulum
ERCC	excise-repair cross-complementary
ERE	estrogen response element
ETL	enteropathy-type T-cell lymphoma
Fab	fragment antigen binding
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
Fc	fragment crystallizable
FcRN	neonatal Fc receptor
FDA	Food and Drug Administration
FEC	chemotherapy with fluorouracil, epirubicin, and cyclophosphamide
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FHIT	fragile histone triad
FISH	fluorescence <i>in situ</i> hybridization
FOBT	fecal occult blood testing
FPGS	folylpolyglutamate synthetase
Fr	framework
FSH	follicle-stimulating hormone
FTI	farnesyltransferase inhibitor
GBM	glioblastoma multiforme
GBP	GSK3-binding protein
GCV	ganciclovir
GDEPT	gene-directed enzyme prodrug therapy
GDP	guanosine 5'-diphosphate
GEF	GTP exchange factor
GERD	gastroesophageal reflux disorder
GIST	gastrointestinal stromal tumor
GJIC	gap junctional intercellular communication
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPAT	gene prodrug activation therapy
GPCR	G-protein-coupled receptor
GRE	glucocorticoid response element
GSH	glutathione
GSK	glycogen synthase kinase
GST	glutathione-S-transferase
GTP	guanosine 5'-triphosphate

GVHD	graft-vs-host disease
HACA	human antichimeric antibody
HADA	human antidrug antibody
HAHA	human antihuman antibody
HAMA	human antimouse antibody
HAT	histone acetyltransferase
HATA	human antitoxin antibody
HbsAg	hepatitis B surface antigen
HBV	hepatitis B virus
hCG	human chorionic gonadotropin
HD	Hodgkin's disease
HDAC	histone deacetylase
HGF	hepatocyte growth factor
Hh	hedgehog
HIF	hypoxia-inducible factor
HLH	helix-loop-helix
HNPCC	hereditary nonpolyposis colorectal cancer
HPLC	high performance liquid chromatography
HNSCC	head-and-neck squamous cell carcinoma
HPV	human papilloma virus
HRE	hypoxia responsive element
HSV	herpes simplex virus
IAP	inhibitor of apoptosis
IARC	International Agency for Research on Cancer
IC <sub>50</sub>	inhibitory concentration, 50%
ICAT	isotype-coded affinity tag
IDC	infiltrating ductal carcinoma
IEN	intraepithelial neoplasia
IFN	interferon
Ig	immunoglobulin
IgH	heavy chain
IgL	light chain
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IGCCCG	International Germ Cell Cancer Collaborative Group
IHC	immunohistochemistry
IL	interleukin
iNOS	inducible nitrous oxide
IP3	inositol triphosphate
IPG	immobilized pH gradient
IRS	insulin receptor substrate
ITAM	immunoreceptor tyrosine-based activation motif

ITIM	immunoreceptor tyrosine-based inhibitory motif
iv	intravenous
J	joining
JAK	Janus kinase
JNK	Jun N-terminal kinase
kb	kilobase
kDa	kilodalton
KIR	killer immunoglobulin-like receptor
KO	knockout
LAK	lymphokine-activated killer
LAP	latency-associated promoter
LATS	linker for activation of T-cells
LBD	ligand-binding domain
LCM	laser capture microdissection
LDH	lactate dehydrogenase
LD-PCR	long-distance polymerase chain reaction
LH	lutein hormone
LIF	leukemia inhibitory factor
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
LPS	lipopolysaccharide
LRP	lung-resistance protein
MAb	monoclonal antibody
MAHA	monkey antihuman antibody
MALT	mucosa-associated lymphoid tissue
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
Mb	megabase
MCL	mantle cell lymphoma
MCP	monocyte chemotactic protein
MCR	mutation cluster region <i>or</i> minor cluster region
M-CSF	macrophage colony-stimulating factor
MDR	multidrug resistance
MDS	myelodysplastic syndromes
MEF	mouse embryo fibroblast
MEN	multiple endocrine neoplasia
MGDF	megakaryocyte growth and development factor
MHC	major histocompatibility complex
MI	microsatellite instability
MIN	microsatellite instability
MLL	mixed lineage leukemia

MMP	matrix metalloproteinase or microsatellite-mutator phenotype
MMR	mismatch repair
MMTV	mouse mammary tumor virus
MPF	maturation-promoting factor
MPP	methylpiperidiopyrozole
MRI	magnetic resonance imaging
MRP	multidrug resistance–associated protein
MSI	microsatellite instability
MSP-PCR	methylation-specific polymerase chain reaction
MTC	major translocation cluster
MTD	maximum tolerated dose
MT-MMP	membrane-type metalloproteinase
MVD	microvessel density
MXR	mitoxantrone-resistance protein
NBS	Nijmegen breakage syndrome
NCAM	neural cell adhesion molecule
NCI	National Cancer Institute
NCoR	nuclear receptor corepressor
NDP	nucleoside diphosphate
NER	nucleotide-excision repair
NF- $\kappa$ B	nuclear factor- $\kappa$ B
NGF	nerve growth factor
NHEJ	nonhomologous end joining
NHL	non-Hodgkin's lymphoma
NIH	National Institutes of Health
NK	natural killer
NMR	nuclear magnetic resonance
NMSC	nonmelanoma skin cancer
NOCEDP	National Ovarian Cancer Early Detection Program
NR	nuclear receptor or nuclear recognition
NSAID	nonsteroidal anti-inflammatory drug
NSCLC	non–small cell lung cancer
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAI	plasminogen activator inhibitor
PAP	prostatic acid phosphatase
PBC-ALL	precursor B-cell acute lymphoblastic leukemia
PBSC	peripheral blood stem cell
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction

PD-ECGF	platelet-derived epidermal cell growth factor
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PEI	chemotherapy with polyethyleneimines
PET	positron emission tomography
PG	prostaglandin
P-gp	P-glycoprotein
PgR	progesterone receptor
Ph	Philadelphia chromosome
PH	pleckstrin homology
PhIAT	phosphoprotein isotype-coded affinity tag
PI3K	phosphatidylinositol 3' kinase
PIN	prostatic intraepithelial neoplasia
PIP <sub>2</sub>	phosphatidylinositol 3,4-biphosphate
PIP <sub>3</sub>	phosphatidylinositol 3,4,5-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
PPT	propyl pyrazole triol
PR	progesterone receptor
pRb	retinoblastoma protein
PSA	prostate-specific antigen
PTB	phosphotyrosine-binding
Ptc	patched
PTH	parathyroid hormone
Rb	retinoblastoma protein
RECIST	Response Evaluation Criteria in Solid Tumors
RER	replication error repair
RES	reticuloendothelial system
REV-T	reticuloendotheliosis vs strain T
RFLP	restriction fragment length polymorphism
rHu	recombinant human
rHuCSF	recombinant human colony-stimulating factor
rHuEPO	recombinant human erythropoietin
rHuG-CSF	recombinant human granulocyte colony-stimulating factor
rHuGM-CSF	recombinant human granulocyte-macrophage colony-stimulating factor
RNAi	RNA interference
RNA-ISH	RNA <i>in situ</i> hybridization
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction

SAGE	sequential analysis of gene expression
sc	subcutaneous
SCE	sister chromatid exchange
SCF	stem cell factor
SCID	severe combined immunodeficiency
SCLC	small cell lung cancer
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELDI	surface-enhanced laser desorption ionization
SELECT	Selenium and Vitamin E Cancer Prevention Trial
SEREX	serologic identification of antigen by recombinant expression cloning
SERM	selective estrogen receptor modulator
SF	scatter factor
SGK	serum and glucocorticoid kinase
SH2	Src-Homology 2
Smo	smoothened
SNP	single nucleotide polymorphism
Sos	son-of-sevenless
SPF	S-phase fraction
SRC	steroid receptor coactivator
SRF	serum response factor
SSB	single-strand break
SSCP	single-strand conformation polymorphism
SSR	single sequence repeat
STAT	signal transducers and activators
SWOG	Southwest Oncology Group
T-ALL	T-cell acute lymphocytic leukemia
TAM	tumor-associated macrophage
TAP	transporter-associated with antigen processing
TCF	ternary complex factor
TCR	T-cell receptor <i>or</i> transcription-coupled repair
TGGE	temperature-gradient gel electrophoresis
TGF	transforming growth factor
THC	5,11- <i>cis</i> -diethyl-5,6,11,12-tetrahydrochrysene-2,8-diol
TIL	tumor-infiltrating lymphocyte
TILN	tumor-infiltrated lymph node
TIMP	tissue inhibitor of matrix metalloproteinase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TNM	staging of cancer based on primary tumor, regional lymph node involvement, and distant metastases

TP	thymidine phosphorylase
TPO	thrombopoietin
TRAIL	TNF-related apoptosis inducing ligand
TS	thymidylate synthase
TSG	tumor suppressor gene
TSH	thyroid-stimulating hormone
TTGE	temperature gradient gel electrophoresis
UBF	upstream binding factor
ULBP	UL16-binding proteins
uPA	urokinase plasminogen activator
uPAR	uPA receptor
UTR	untranslated region
UV	ultraviolet
V	variable
VCR	variant cluster region
VDEPT	virus-directed enzyme prodrug therapy
VDJ	variable diversity joining
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VNR	vitronectin receptor
VPF	vascular permeability factor
$V_{ss}$	volume of steady state
WHO	World Health Organization
XP	xeroderma pigmentosum

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